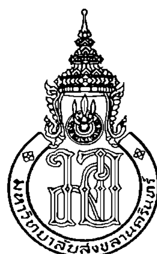

Properties and Changes during Fermentation of Salted Shrimp Paste as Affected by Quality of Raw Material and Selected Halophilic Bacteria

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A Thesis Submitted in Partial Fulfillment of the Requirements for the Degree of Doctor of Philosophy in Food Science and Technology

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Thesis Title	Properties and changes during fermentation of salted shrimp paste as affected by quality of raw material and selected halophilic bacteria
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I hereby certify that this work has not been accepted in substance for any degree, and is not being currently submitted in candidature for any degree.

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ชื่อวิทยานิพนธ์ ผลของคุณภาพของวัตถุดิบและแบคทีเรียชอบเค็มที่คัดเลือกได้ต่อสมบัติและการเปลี่ยนแปลงระหว่างหมักกะปิ
ผู้เขียน นางสาวจักรสุมา พงศ์เศรษฐ์กุล
สาขาวิชา วิทยาศาสตร์และเทคโนโลยีอาหาร
ปีการศึกษา 2560

บทคัดย่อ

จากการตรวจวิเคราะห์ตัวอย่างกะปิทางการค้าทั่วประเทศไทย จำนวน 11 ตัวอย่าง พบว่ากะปิมีองค์ประกอบทางเคมีแตกต่างกัน โดยมีโปรตีนเป็นองค์ประกอบหลัก (ร้อยละ 29.44-53.27 คิดบนฐานน้ำหนักแห้ง) ปริมาณเกลือและค่าวอเตอร์แอคติวิตี้ของกะปิอยู่ในช่วงร้อยละ 22.77-35.47 และ 0.695-0.774 ตามลำดับ ปริมาณฟอร์มอลไนโตรเจน (11.96-22.87 มิลลิกรัมไนโตรเจน/กรัมตัวอย่าง) และระดับการไฮโดรไลซิส (ร้อยละ 12.68-20.76) ที่แตกต่างกันแสดงให้เห็นถึงการย่อยสลายของสายเปปไทด์ที่แตกต่างกันของตัวอย่าง กะปิมีสีและปริมาณแคโรทีนอยด์ทั้งหมด (0.54-1.97 มิลลิกรัม/กรัมตัวอย่าง) แตกต่างกัน รวมทั้งมีองค์ประกอบกรดอะมิโนและสารระเหยได้แตกต่างกัน โดยมีกรดอะมิโนกลูตามิก/กลูตามีนและอนุพันธ์ไพราซีนเป็นองค์ประกอบหลักที่พบในทุกตัวอย่าง นอกจากนี้กะปิทุกตัวอย่างมีกิจกรรมการต้านออกซิเดชัน ดังนั้น กะปิจึงจัดเป็นเครื่องปรุงที่อุดมไปด้วยโปรตีนและคุณค่าทางโภชนาการ

เมื่อศึกษาเปรียบเทียบกุ้งเคย 2 สายพันธุ์คือ *Acetes vulgaris* และ *Macrobrachium lanchesteri* เพื่อนำมาใช้เป็นวัตถุดิบทางเลือกสำหรับผลิตกะปิ พบว่ากุ้งทั้ง 2 สายพันธุ์ประกอบด้วยเอนไซม์กลุ่มซีรินโปรติเอสเป็นหลักและถูกยับยั้งด้วย PMSF และ SBTI เอนไซม์โปรตีเอสของกุ้ง *A. vulgaris* และ *M. lanchesteri* มีกิจกรรมสูงสุดที่พีเอช 7 อุณหภูมิ 60°ซ และพีเอช 8 อุณหภูมิ 60°ซ ตามลำดับ เอนไซม์โปรตีเอสจากกุ้งทั้ง 2 สายพันธุ์มีกิจกรรมลดลงเมื่อความเข้มข้นของเกลือเพิ่มสูงขึ้น (ร้อยละ 0-30) กิจกรรมของเอนไซม์โปรตีเอส ทริปซิน และไคโมทริปซินสามารถตรวจพบตลอดกระบวนการผลิตกะปิ กิจกรรมเหล่านี้ลดลงในขั้นตอนการหมักเกลือ แต่เพิ่มสูงขึ้นเรื่อย ๆ เมื่อระยะเวลาการหมักเพิ่มสูงขึ้น การเปลี่ยนแปลงดังกล่าวสอดคล้องกับการเพิ่มขึ้นของปริมาณเปปไทด์ที่ละลายในกรดไตรคลอโรแอซิติกและการจางหายของแถบโปรตีนไมโอซินและแอคตินเอนไซม์โปรตีเอสเหล่านี้มีส่วนเกี่ยวข้องกับการย่อยสลายโปรตีนของกุ้งระหว่างการผลิตกะปิ

ระยะเวลาการเก็บรักษาหลังการตายของกุ้งก่อนนำกุ้งมาหมักเกลือมีผลต่อคุณภาพกะปิ กุ้งทั้ง 2 สายพันธุ์เกิดการเสื่อมเสียเมื่อวางทิ้งไว้ที่อุณหภูมิห้อง (28-30°ซ) บ่งชี้ได้จากการเพิ่มขึ้นของค่าพีเอช ปริมาณด่างระเหยได้ทั้งหมด ปริมาณไตรเมทิลเอมีน ปริมาณ TBARS และ

ปริมาณจุลินทรีย์ทั้งหมด กะปิมีสีคล้ำมากขึ้น รวมทั้งมีปริมาณสารระเหยได้และกิจกรรมการต้านออกซิเดชันเพิ่มสูงขึ้นเมื่อกุ้งที่ใช้เป็นวัตถุดิบมีระยะเวลาการเก็บรักษาหลังการตายสูงขึ้น อย่างไรก็ตาม คะแนนด้านกลิ่นและความชอบรวมของกะปิลดลงเมื่อใช้กุ้งไม่สดเป็นวัตถุดิบ (เมื่อระยะเวลาการเก็บรักษาหลังการตายของกุ้ง *A. vulgaris* และ *M. lanchesteri* มากกว่า 6 และ 12 ชั่วโมง ตามลำดับ)

จากการเปรียบเทียบคุณภาพของกะปิที่ผลิตจากกุ้ง *A. vulgaris* (KA) และ *M. lanchesteri* (KM) พบว่ากะปิทั้งสองตัวอย่างมีองค์ประกอบทางเคมี กายภาพและคุณสมบัติทางประสาทสัมผัสแตกต่างกัน กะปิ KM มีปริมาณโปรตีนสูงกว่า ในขณะที่มีปริมาณไขมันต่ำกว่าเมื่อเปรียบเทียบกับกะปิ KA อย่างไรก็ตาม กะปิ KA มีระดับการย่อยสลาย ปริมาณเพอร์ออกไซด์ ปริมาณ TBARS และการเกิดสีน้ำตาลสูงกว่ากะปิ KM ซึ่งบ่งชี้ถึงการย่อยสลายโปรตีน การเกิดปฏิกิริยาเมลลาร์ดและการเกิดออกซิเดชันของไขมันที่สูงกว่าของตัวอย่างกะปิ KA นอกจากนี้ กิจกรรมการต้านออกซิเดชันในตัวอย่างดังกล่าวมีค่าสูงกว่าเช่นกัน ดังนั้น กุ้ง *A. vulgaris* มีศักยภาพในการใช้เป็นวัตถุดิบทางเลือกสำหรับการผลิตกะปิสูงกว่า เมื่อเปรียบเทียบกับกุ้ง *M. lanchesteri*

จากการศึกษาการเปลี่ยนแปลงทางเคมีและจุลินทรีย์ของกุ้ง *A. vulgaris* ระหว่างการผลิตกะปิ ประกอบด้วยขั้นตอนการหมักเกลือ การตากแดด และการหมัก พบว่าพีเอชของกุ้งสดมีค่าลดลงจนถึงวันที่ 10 ของการหมัก และเพิ่มสูงขึ้นอย่างต่อเนื่องเมื่อระยะเวลาการหมักเพิ่มขึ้น สีของกะปิเปลี่ยนเป็นสีน้ำตาลหรือคล้ำมากขึ้นเมื่อระยะเวลาการหมักเพิ่มขึ้น การเปลี่ยนแปลงองค์ประกอบอื่น ๆ เกิดขึ้นตลอดกระบวนการผลิตกะปิ ซึ่งส่วนใหญ่เกิดในขั้นตอนการหมักเป็นหลัก ประกอบด้วย การย่อยสลายโปรตีนและสารประกอบอะดีโนซีนไตรฟอสเฟต การเกิดออกซิเดชันของไขมัน และการผลิตสารระเหยได้ การเปลี่ยนแปลงดังกล่าวมีผลต่อคุณลักษณะสุดท้ายของกะปิ โดยเฉพาะด้านรสชาติและกลิ่นรส

กุ้ง *A. vulgaris* มีปริมาณน้ำมันร้อยละ 4.21 คิดบนฐานน้ำหนักแห้ง น้ำมันดังกล่าวส่วนใหญ่ประกอบด้วยไตรเอซิลกลีเซอรอล (ร้อยละ 28.03) และสเตอรอล (ร้อยละ 24.03) องค์ประกอบดังกล่าวมีปริมาณลดลงตลอดกระบวนการผลิตกะปิ ในขณะที่ปริมาณกรดไขมันอิสระเพิ่มสูงขึ้นและกลายเป็นองค์ประกอบหลักในผลิตภัณฑ์กะปิ (ร้อยละ 49.29) ไตรเอซิลกลีเซอร์รอลและกรดไขมันอิสระประกอบด้วยกรดไขมันชนิดอิ่มตัวและกรดไขมันชนิดไม่อิ่มตัวที่มีพันธะคู่ 1 พันธะเป็นกรดไขมันหลัก กรดไขมันชนิดไม่อิ่มตัวที่มีพันธะคู่หลายพันธะ โดยเฉพาะกรดอีโคซะเพนตะอีโนอิก (C20:5(n-3)) และกรดโดโคซะเฮกซะอีโนอิก (C22:6(n-3)) พบมากในฟอสฟาติดิลเอตทาโนลามีนและฟอสฟาติดิลโคลีน การย่อยสลายไขมันและการเกิดออกซิเดชันของไขมันเกิดขึ้นตลอดกระบวนการผลิตกะปิ บ่งชี้ได้จากการเพิ่มสูงขึ้นของปริมาณ

เพอร์ออกไซด์ ปริมาณ TBARS และปริมาณกรดไขมันอิสระ การเปลี่ยนแปลงดังกล่าวมีผลต่อคุณลักษณะของกะปิ โดยเฉพาะด้านกลิ่นรส

จากการศึกษาคุณลักษณะเชื้อ *Bacillus* spp. K-C3 ที่คัดแยกจากกะปิทางการค้า พบว่า การเจริญเติบโตและการผลิตเอนไซม์ที่หลั่งออกมานอกเซลล์ประกอบด้วยเอนไซม์โปรตีเอส ไลเปส และไคติเนสเกิดขึ้นสูงสุดที่พีเอช 6-8 อุณหภูมิ 25-35°ซ ในสภาวะที่มีเกลือร้อยละ 10 เมื่อศึกษาเปรียบเทียบคุณลักษณะของกะปิที่เติม *Bacillus* spp. K-C3 ที่ระดับ 10^2-10^6 CFU/กรัมตัวอย่าง (คิดบนฐานน้ำหนักแห้ง) กับกะปิชุดควบคุม (ไม่มีการเติมหัวเชื้อ) จากการตรวจติดตามด้วยเทคนิคพีซีอาร์-ดีจีจีอี พบว่าเชื้อ *Bacillus* spp. K-C3 สามารถตรวจพบได้ทุกขั้นตอนตลอดกระบวนการผลิตกะปิ กะปิที่มีการเติมหัวเชื้อมีกิจกรรมการย่อยสลายโปรตีน ไขมัน และไคตินสูงกว่า สอดคล้องกับปริมาณเปปไทด์ที่ละลายในกรดไตรคลอโรแอซิติก ระดับการไฮโดรไลซิส และปริมาณกรดไขมันอิสระที่สูงกว่าเมื่อเทียบกับกะปิชุดควบคุม อัตราการหมักที่สูงกว่าในตัวอย่างกะปิที่มีการเติมหัวเชื้อส่งผลโดยตรงต่อคุณลักษณะของกะปิ ซึ่งประกอบด้วยการเกิดสีน้ำตาล การเกิดออกซิเดชันของไขมัน กิจกรรมการต้านออกซิเดชัน และการสร้างสารระเหยได้ ดังนั้นการเติมเชื้อ *Bacillus* spp. K-C3 ลงในกุ้ง *A. vulgaris* ระหว่างการผลิตกะปิมีผลทำให้ระยะเวลาการหมักลดลงและสามารถปรับปรุงคุณภาพกะปิ

Thesis Title Properties and changes during fermentation of *Kapi*, salted shrimp paste, as affected by quality of raw material and selected halophilic bacteria

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Major Program Food Science and Technology

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ABSTRACT

Eleven commercial *Kapi*, obtained from various places of Thailand, were characterized. The samples had varying compositions, but protein constituted as the major component (29.44-53.27%, dry weight basis). Salt content and A_w of all samples were in the range of 22.77-35.47% and 0.695-0.774, respectively. Various formal nitrogen contents (11.96-22.87 mg N/g sample) and degree of hydrolysis (DH) (12.68-20.76%) suggested the varying cleavage of peptides among the samples. The samples had different colors with different total carotenoid contents (0.54-1.97 mg/g sample). Amino acid composition and volatile compounds were also varied. Glu/Gln and pyrazine derivatives were dominant in all samples. Moreover, all samples also possesses antioxidative activities. Thus, *Kapi* could be a good source of protein and served as the nutritious condiment.

When small shrimp *Acetes vulgaris* and *Macrobrachium lanchesteri*, used as alternative sources for *Kapi* production, were characterized, both shrimp mainly contained serine proteases, which were strongly inhibited by PMSF and SBTI. Maximal proteolytic activity of *A. vulgaris* and *M. lanchesteri* was found at pH 7, 60°C and pH 8, 60°C, respectively. Activity of both proteases decreased with increasing NaCl concentration (0-30%). During *Kapi* production, protease, trypsin and chymotrypsin activities were detectable throughout all processes. Activities were decreased when salting was implemented but continuously increased with increasing fermentation time. Those changes coincided with increasing TCA-soluble peptide content and disappearance of myosin heavy chain and actin. Those proteases more likely involved in proteolysis of shrimp during *Kapi* production.

Post-mortem storage time of both shrimp prior to salting affected the quality of resulting *Kapi*. Both shrimp underwent deterioration when stored at room temperature (28-30°C) as indicated by the increases in pH, total volatile base (TVB),

trimethylamine (TMA) contents, thiobarbituric acid reactive substances (TBARS) and total viable count (TVC). Resulting *Kapi* became darker and had higher intensity of volatiles and antioxidative activities when shrimp used as raw material were stored for a longer time. However, odor- and overall-likeness scores of *Kapi* were decreased when unfresh shrimp (more than 6 h and 12 h of post-mortem storage time for *A. vulgaris* and *M. lanchesteri*, respectively) were used.

Quality of *Kapi* produced from *A. vulgaris* (KA) and *M. lanchesteri* (KM) were comparatively examined. Both samples had different chemical compositions, physical and sensory properties. KM had the higher protein content but lower fat content, compared with KA ($p < 0.05$). However, KA showed the higher DH, peroxide value (PV), TBARS value as well as browning intensity (A_{420}), compared with KM. This indicated the higher protein degradation, Maillard reaction as well as lipid oxidation occurred in KA. The higher antioxidative activities were also found in KA sample ($p < 0.05$). Overall, *A. vulgaris* showed higher potential as an alternative raw material for *Kapi* production, in comparison with *M. lanchesteri.*

Chemical and microbiological changes of shrimp *A. vulgaris* during *Kapi* production including salting, drying and fermentation were monitored. The pH decreased within the first 10 days of fermentation and continuously increased as fermentation progressed. Color of *Kapi* turned to be browner or darker as fermentation time increased ($p < 0.05$). Several changes of *Kapi* components took place throughout all processes but mainly occurred during fermentation period. Those included degradation of proteins and adenosine triphosphate (ATP), lipid oxidation as well as the formation of volatiles. Those changes played a role in the characteristic of resulting *Kapi,* especially taste or flavor.

A. vulgaris containing 4.21% lipid (dry weight basis), which mainly comprised triacylglycerol (TAG) (28.03%) and sterol (ST) (24.03%). Both TAG and ST gradually decreased during *Kapi* production, while free fatty acid (FFA) increased and became dominant in the final product (49.29%). TAG and FFA fractions mainly consisted of saturated and monounsaturated fatty acids. Polyunsaturated fatty acids (PUFAs), especially eicosapentaenoic acid (EPA) (C20:5(n-3)) and docosahexaenoic acid (DHA) (C22:6(n-3)), were accumulated in phosphatidylethanolamine (PE) and phosphatidylcholine (PC) fractions. During *Kapi* production, lipolysis and lipid

oxidation occurred throughout all processes as evidenced by increases in PV, TBARS value and FFA content. Those changes might partially contribute to the *Kapi* characteristics, especially flavor.

Bacillus spp. K-C3, isolated from commercial *Kapi*, was characterized for its growth and production of extracellular enzymes including protease, lipase and chitinase. The maximal growth and enzyme secretion of this strain were obtained at pH 6-8, 25-35°C in the presence of 10% NaCl. *Kapi* inoculated with *Bacillus* spp. K-C3 at the level of 10^2-10^6 CFU/g dry weight sample were characterized throughout the processes, compared with naturally fermented *Kapi.* Based on polymerase chain reaction-denaturing gradient gel electrophoresis (PCR-DGGE) result, *Bacillus* spp. K-C3 bands were detected in all steps of *Kapi* production, indicating that this strain could survive throughout the entire process. All inoculated samples exhibited the higher proteolytic, lipolytic and chitinolytic activities, corresponding well with the higher TCA-soluble peptide, DH and FFA content of the final products, compared to the control. The greater rate of fermentation in the inoculated samples directly affected characteristics of *Kapi* including the brown color development, lipid oxidations, antioxidative activities as well as formation of volatiles. Therefore, inoculation of shrimp *A. vulgaris* with *Bacillus* spp. K-C3 seemed to be the potential means to accelerate the fermentation and yielded *Kapi* with the preferable characteristics.

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CHAPTER 1

INTRODUCTION AND REVIEW OF LITERATURE

1.1 Introduction

Kapi, a typical traditional salted shrimp paste of Thailand, has been widely consumed as a condiment in several Thai foods. Generally, *Kapi* is produced by mixing 1 part of salt with 3-5 parts of shrimp. The mixture is salted, pounded and spread out on the ground to dry with sunlight. The paste is compacted and allowed to ferment in anaerobe condition at ambient temperature (25-30°C) for at least 1 month or longer until the typical aroma is fully developed (Faithong *et al.,* 2010). The color of *Kapi* varies from a pinkish or purplish grey to a dark greyish brown. The consistency of *Kapi* also ranges from soft and pasty to dry and hard. *Kapi* can be stored for several months (Phithakpol, 1993). In general, type of raw material used, shrimp or krill/salt ratio, fermentation process and time are different, depending on regions or countries. Those factors contribute to the different characteristics and properties of *Kapi* (Peralta *et al.,* 2008).

Kapi is traditionally prepared from the planktonous krill (*Mesopodopsis orientalis*). However, stocks of krill, have decreased by 3% per year over the last decade (Meland and Willassen, 2007). Alternative raw material for *Kapi* production has been searched. Small shrimp belonging to *Acetes vulgaris* has become promising for *Kapi* production in the southern part of Thailand, owing to its availability (Faithong *et al.,* 2010). Recently, another species, *Macrobrachium lanchesteri*, a by-catch from commercial fishing, has been utilized for *Kapi* production because of its abundance, low price, and all year round availability, especially in the southern part of Thailand. Generally krill has the higher proteolysis than shrimp (Bustos *et al.,* 1999). Therefore, different shrimp with different endogenous proteases related with varying autolysis may determine the characteristics and properties of *Kapi.*

During fermentation, biochemical and microbiological changes occur in shrimp or *Kapi.* Proteolysis is an essential biochemical reaction take place during the

fermentation of *Kapi.* It influences both texture and flavor developed. This is associated with the formation of several low molecular weight compounds, including peptides, amino acids, aldehydes, organic acids and amines (Peralta *et al.,* 2008). Proteolysis is induced by endogenous proteases in shrimp/krill as well as proteases produced by halophilic bacteria surviving under high salt condition (Gildberg and Stenberg, 2001). Lipolysis during fermentation is also important for odor or flavor of *Kapi.* Free fatty acids (FFA) are liberated and further undergo oxidation. Oxidation products such as aldehydes and ketones are responsible for the development of flavor or aroma of *Kapi* (Lizaso *et al.,* 1999). Fermentation process normally takes at least 1 month, in which the desired flavor, color and aroma are developed (Phithakpol, 1993).

Due to the inconsistency quality of *Kapi* produced by traditional process, the use of starter culture can bring about the repeatability of *Kapi* production and improve the sensory characteristics as well as microbiological quality of the product. The inoculation of proteolytic and lipolytic producing bacteria to produce *Kapi* might shorten fermentation time and enhance the taste and flavor of the final product. As a consequence, prime quality *Kapi* can be manufactured with comparable quality to that typically produced from krill. Furthermore, *Kapi* may contain bioactive components, which can be beneficial for health e.g. antioxidative activity, antimicrobial activity and ACE inhibitory activity. With the starter culture, bioactivities of product can be also augmented.

The information on biochemical and microbiological changes during fermentation of *Kapi* can provide better understanding on the major factors affecting the final quality of the product. *Kapi* production using the alternative raw material can be achieved. The use of starter culture can be a potential means in producing *Kapi* possessing the desirable characteristics with the shorter processing time.

1.2 Review of Literature

1.2.1 Fermented foods

Fermentation is one of the oldest and most widely used food preservation methods in households and small-scale food industries as well as in large enterprises (Phithakpol, 1993). Fermented food is foodstuff, which has gone through the

fermentation process by microorganisms such as mold, bacteria or yeast. Fermented foods are involved by the action of microorganisms or enzymes, which cause desirable biochemical changes and significant modification to the food (Phithakpol, 1993). Fermented foods are of great significance because they are nutritive and have a wide diversity of flavors, aromas and textures. Development of flavor can vary, depending on raw materials, starter cultures and processing conditions (Lertprakobkit, 2011).

The fermentation of aquatic products is primarily concerned with the controlled degradation of proteinaceous materials into more stable or at least different tasting products. Seafood, including shrimp, is highly perishable and the quality deterioration is usually dominated by microorganisms. Normally, shellfish undergo the deterioration more rapidly than fish for a number of reasons. Firstly, they are smaller, and spoil more rapidly than larger ones. Secondly, and more importantly, the gut is usually not removed immediately after capture; hence post-mortem autolytic changes will occur faster. A third reason is that the chemical composition of shellfish tissue is different and it contains a lot of non-protein nitrogenous compounds that encourage more rapid spoilage (Phithakpol, 1993). For traditional processes, the degradation is controlled primarily by the addition of salt. Control of the oxygen content during fermenting also helps in determining the final product characteristics (Lertprakobkit, 2011). The most common putrefactive microorganisms on fishery product are inhibited at salt contents above 6 to 8 percent (Lertprakobkit, 2011). Fermented process could be different by these variables among process including (1) raw material and salt microflora, (2) endogenous proteases of raw material and some characteristic, (3) the presence of visceral enzymes, (4) condition during the fermentation process, (5) temperature, (6) pH of the fermentation mixture, (7) presence or absence of oxygen, (8) nutritional state of the raw material, (9) the presence of carbohydrates, and (10) fermentation process time (Phithakpol, 1993). Process duration is of particular importance in products containing fish or seafood, because these will eventually liquefy if the process is allowed to proceed for a long time without controlling the moisture content (Mackie *et al.,* 1982).

1.2.2 Fermented fishery products

The term 'fermented fishery products' is used to describe the products of freshwater and marine finfish, shellfish and crustaceans that are processed by the combined action of fish enzymes and bacterial enzymes with salt to cause fermentation, thereby preventing putrefaction (Ruddle and Ishige, 2010). The enzymes induce textural change and assist in the production of flavor while the bacteria are involved in developing the aroma and flavor for fish fermentation (Beddows, 1979). Fish preservation occurs in areas where there is a seasonal abundance of local fish. The freshwater fish species naturally occurring in local hydrological systems have been fermented. The fermentation process first developed among sedentary farmers and only where salt was easily obtained. Fish fermentation has been developed in a focal regions of continental East Asia and Southeast Asia (Ruddle and Ishige, 2010). Such a wide range of fermented fish is produced in East Asia. They are classified based on the nature of the final product and the preparation method used.

1.2.2.1 Classification of Thai fermented fishery products

Thai traditional salt/fish fermented products are mostly produced according to family tradition and local geographic preferences. Therefore, the differences are governed by raw materials, ingredients and production methods (Lertprakobkit, 2011). The classification of Thai traditional fermented foods can be done according to various methods based on final appearance, main processing techniques, ingredients used as well as type of raw materials (Phithakpol, 1993; Saisithi, 1994).

Vinderola *et al.* (2008) broadly classified Thai fermented food based on raw materials used. Those include (1) Fishery products (*Nampla* [fish sauce], *Kapi* [shrimp paste] and *Budu*), (2) Meat products (Nham [fermented pork sausage], *Saikrogprieo* [fermented sausage] and *Mam* [fermented beef/pork sausage or beef/pork liver]), (3) Plant products (*Nawmaidong* [fermented bamboo shoot], *Phakgarddong* [pickled green mustard], *Miang* [fermented tea leaves]), (4) Grains and cereal products (*Khaomak* [fermented glutinous rice], *Khanom-jeen* [fermented rice noodle], soy sauce, *Namkhao* or *Krachae* or *Ou* [the rice wine]).

Traditional fermented products can be classified based on the type of substrate and source of enzyme into 3 groups (Saisithi, 1994). Those are shown as follows:

1) Group I products

Products are processed from fish/salt mixture and hydrolyzed by the combined action of fish and bacterial enzymes. The examples are *Nam-pla*, *Pla-ra* or *Ka-pi.* Both fish sauce and fish paste are prepared from whole fish. The addition of salt to fish is to prevent the spoilage caused by microorganisms. The enzymes from both fish and microorganisms are responsible for hydrolysis. Firstly, fermentation starts with fish enzymes, especially digestive proteases, resulting in the small peptides. The resulting peptides are used as the substrate for the microorganisms, which play an important role in the further hydrolysis as well as flavor development.

2) Group II products

Products, belonging to this category, are produced from fish/salt/ carbohydrate mixture partially hydrolyzed by bacterial enzyme. Products include *Pla-chom, Pla-som* and *Som-fug.* Lactic acid bacteria play an essential role in flavor and acid taste development in the products. There are a variety of fermented products in this category. Fish can be prepared in different forms, such as the whole fish, pieces and mince. Carbohydrate is usually added in the form of cooked rice. The ratio of salt to fish is 1:3 or 1:4. Salting and fermenting time vary from 1 to 2-3 days (Lertprakobkit, 2011). After salting and fermenting, cooked rice is mixed and fermentation is allowed to take place, generally for 2-3 days. Carbohydrate is the carbon source for microorganisms, especially lactic acid bacteria. The acids formed are the main preservatives and contribute to the flavor and taste.

3) Group III products

Products under this category are prepared from fish/salt mixture, hydrolyzed mostly by fish enzymes in the presence of carbohydrate fermented by yeast and mold (Lertprakobkit, 2011). *Pla-chao* and *Kung-chao* are the examples of this group. Prior to processing, fish or shrimp are prepared in the same manner with *Pla-chom* and *Kung-chom.* However, the fish or shrimp is mixed with salt with the ratio of 3:1 and the mixture is left for 2 days. The liquid formed is drained and the fish (3 parts) is mixed with *Khao-mak* (alcoholic fermented steamed rice). The mixture is left for 10-

20 days for fermentation. The products have a sweet, sour and salty taste with slightly alcoholic flavor.

Fermented fishery products can be categorized according to main processing techniques and ingredients used (Phithakpol, 1993). Although some products have the similar processing, some ingredients used can be varied, leading to the different characteristics, especially flavor and taste. Different raw materials including fish, shrimp, mollusk, etc. have also provided the variety of products with differences in appearance, flavor, taste as well as other sensorial properties. Based on type of raw materials, the products are divided into 3 groups including fermented shrimp, fermented fish and fermented mussel (Phithakpol, 1993) as shown in Table 1.

Table 1. Raw materials and ingredients of fermented fishery products

Fermented fishery products	Name	Raw materials and ingredients
Fermented shrimp/krill	*Ka-pi*	Shrimp or krill and salt
	Kung-chom	Shrimp, salt, fish sauce and mixed roasted rice
	Naam-kheeuy	Krill and salt
Fermented fish	*Budu* (fish sauce)	Marine fish and salt
	Tai-pla	Viscera of fish and salt
	Pla-ra	Freshwater fish, salt, roasted rice and rice bran
	Pla-chao	Freshwater fish and *Khao-mak* (alcoholic fermented steamed rice)
	Pla-som	Freshwater fish, salt, steamed rice and/or sticky rice and garlic
	Som-fug	Freshwater fish mince, salt, ground steamed rice and minced garlic
	Pla-chom	Freshwater fish, salt and roasted rice
	Kem-buk-nud	Freshwater fish mince, salt and pine apple
Fermented mussel	*Hoi-dong*	Mussel, salt, tamarind, sugar and coconut juice

Source: Modified from Phithakpol (1993).

1.2.2.2 Microbiology of fermented fishery products

Marine fish in its natural environment has its own microflora on its body. These microorganisms, as well as the enzymes in the tissues of the fish, bring about putrefactive changes in fish when it dies (Phithakpol, 1993). Additionally, the microorganisms generally present in the salt used for salting also contribute to the degradative changes in the fish (Lertprakobkit, 2011). Basically, salt has been

introduced to lower water activity of fish subjected to fermentation and prevent the spoilage of fish or shellfish (Table 2). The salt concentration may range from 10 to 30% (w/w) in different types and batches of fermented fishery products (Saisithi, 1994). This is likely to have a pronounced influence on the microbial growth and the rate of fermentation, and thereby on the sensory quality and safety of the product. It is therefore of interest to identify the optimal salt concentration, which does not inhibit the growth of the fermenting microorganisms, and in addition contributes positively to the flavor and texture of the product.

Table 2. Growth of microorganisms on fermented fishery products

Water activity (A_w)	NaCl (%)	Microorganisms grooving	
		Pathogens	Spoilage organisms
0.98	<3.5	All known food-borne	Most microorganisms pathogens of concern in foods particularly the gram negative rods
0.98-0.93	3.5-10	*Bacillus cereus, Clostridium botulinum, Salmonella* spp., *Clostridium perfringens, Vibrio parahaemolyticus*	Lactobacilliaceae, Enterobacteriaceae, Bacilliaceae, Micrococcaceae, Molds
0.93-0.85	10-17	*Staphylococcus*	Cocci, yeasts, molds
0.85-0.60	>17	Mycotoxic, xerophilic molds (no mycotoxin is produced at A_w less than 0.80)	Halophilic bacteria, Yeasts, molds

Source: Silliker *et al.* (1980).

All microbial growth is inhibited at water activity (A_w) below 0.60 (Table 2). Halophiles grow optimally at high salt concentrations but are unable to grow in salt-free media. Halophiles have the capacity to balance the osmotic pressure of the environments and resist the denaturing effect of salts (Grant, 2001). Kushner (1993) classified microorganisms based on their requirement for NaCl into five groups, which are non-halophiles (<0.2 M, ~1% salt), slight halophiles (0.2-0.5 M, ~1-3% salt), moderate halophiles (0.5-2.5 M, ~3-15% salt), borderline extreme halophiles (1.5-4.0 M, ~9-23% salt) and extreme halophiles (2.5-5.2 M, ~15-32% salt). Halotolerant organisms grow best without significant amounts of salt but can also grow at concentrations higher than that of sea water (Namwong *et al.,* 2007). In addition,

halophilic bacteria can be found in fermented fish and fishery product such as fish sauce, fermented fish pastes and salted fish. Xerophiles are those organisms which grow rapidly under relatively dry conditions or below A_w of 0.85, while osmophiles can grow under high osmotic pressure (Grant, 2001). Most food-borne bacterial pathogens are not able to grow in an A_w range of 0.98-0.93 (Namwong *et al.,* 2007) as shown in Table 2.

1.2.3 Fermented shrimp products

1.2.3.1 Production of fermented shrimp products

Fermented shrimp products are mainly categorized into sauces, pastes, and lacto-fermented products (Hajeb and Jinap, 2013). It is believed that shrimp paste was originated in the continental Southeast Asia, probably among the Cham and Mon peoples of Indochina (Ruddle and Ishige, 2010). Fermented shrimp paste is known by several names in different regions of Southeast Asia as shown in Table 3. It is called *Belacan* in Malaysia, *Kapi* in Thailand and Cambodia, *Nga-pi* in Myanmar, *Bagoong-alamang* in Philippines, *Terasi* in Indonesia or *Mam* in Vietnam (Hajeb and Jinap, 2013).

Table 3. Fermented shrimp products in Southeast Asian countries

Country	Shrimp sauce	Shrimp paste	Fermented shrimp
Burma	*Ngan pya ye*	*Seisanga-pi /Hmyinnga-pi*	
Cambodia	*Nam tom*	*Kapi / Pra-hoc / Mam-ruoc*	
Indonesia		*Terasiudang*	
Malaysia		*Belacan*	*Cencalok*
Myanmar	*Pazunggampya ye*	*Nga-pi / Seinza / Hmyannga-pi*	
Philippines	*Alamang-patis*	*Bagoong-alamang / Dinailan / Lamayo / Buronghipon*	*Balao-balao*
Thailand	*Nam kapi* *Nam khoei*	*Kapi*	*Jaloo* *Koongsom*
Vietnam	*Nam tom* *Nam mam* *Tom chat*	*Mam ruoc* *Mam tom* *Mam tep*	

Source: Hajeb and Jinap (2013).

As compared to fermented fish products, shrimp pastes have lower salt content. This may be due to the different composition of fish and shrimp. Shrimp has higher water content than fish. Most of people in the countries in this region regularly use shrimp sauce and paste in their daily cooking. Depending on each region, the species of shrimp, the quantity of salt used, and the treatment of raw materials prior to fermentation, can be varied. In order to produce fermented shrimp products, salt is mixed with cleaned fresh or dried shrimp and allowed to be fermented for several months in order to enable the indigenous enzymes to auto-digest the meat and create products with high amino acids content (Ishige, 1993). The enzymatic fermentation of shrimp mediated by indigenous proteases yields short chain peptides and free amino acids which render the typical flavor and taste. Salt is added to prevent deterioration and food poisoning as well as to yield meaty-savory flavor (Steinkraus, 2002). At the end of the process, shrimp paste is usually dried to reduce the moisture content and to produce a semi-solid product. The semi-solid nature of the product means that it needs only a little amount of salt and it has a strong umami taste (Ishige, 1993). The drying process and reduction of moisture also increase the shelf life and flavor intensity of the product. Complete drying would prevent rancidity of the product. The addition of salt would enhance both the flavor and the shelf-life of the shrimp paste (Ishige, 1993).

Fermented shrimp paste is produced using various protocols in different parts of the countries in Southeast Asia. In some parts of Myanmar, Indonesia, and Philippines, shrimp paste is produced without the use of salt. Comminuting the sun dried shrimp without the addition of salt is also practiced in some parts of Thailand. This technique uses epipelagic shrimp to produce an unsalted shrimp paste (Ruddle and Ishige, 2010). The final product of *Bagoong-alamang* produced in some parts of the Philippines, contain the shell of shrimp. If the shell shrimp paste is fermented for a long period of time, the shell eventually decomposes and the product becomes a semi-liquid paste, as in the Zhejiang of China (Ruddle and Ishige, 2010). Fermented shrimp products develop umami taste formed by the degraded products, amino acids, nucleotides and salt. Therefore, such fermented products are also a good source of peptides and amino acids (Rajapakse *et al.,* 2005; Peralta *et al.,* 2008).

Kapi is a typical traditional fermented shrimp paste of Thailand. It is mainly produced from the marine shrimp or krill (*Acetes* or *Mesopodopsis* species),

which are mixed with salt at a ratio of 3-5:1. The moisture content is decreased by sun drying, and then it is thoroughly blended or homogenized to produce semi-solid paste. The paste is fermented for at least 1 month or until the desired flavor is developed (Phithakpol, 1993). *Kapi* is usually used as condiment to enhance the palatability of foods. When *Kapi* is mixed with chilli, it is called *pherik-kapi,* which is also regarded as a favorite spicy condiment in Thailand. *Pherik-kapi* is typically made of raw chilli which is pounded with toasted fermented shrimp paste that has been added with salt, sugar and organic acids. *Kapi* is very rich in umami taste and contain high amounts of free glutamic acid (647 mg/100 g) (Mizutani *et al.,* 1987).

Belacan is a traditional salted and fermented shrimp paste of Malaysia. It is a thick paste with a greyish pink to greyish purple color and it has a strong pungent shrimp taste i.e., buttery, sting taste with a touch of sweetness. *Belacan* is one of the main condiments in Malaysian cuisine and it is usually added as a flavoring ingredient in most local dishes (Mah Hassan, 2008). Shrimp of *Acetes* species and high amount of salt are the main raw materials for production of *Belacan.* Shrimp tissues undergo enzymatic breakdown during the fermentation and bacterial action assist in proteolysis and flavor development. Various techniques used to produce *Belacan* have the impact on composition and quality among products. In Malaysia, considerable amount of shrimp paste are produced in fishing villages using traditional method via starter culture and microbial fermentation. Dried shrimp is usually used for paste production. Salt is added with different ratio (5-20%) and mixed thoroughly. The darker colored shrimp paste is produced after it is being fermented for one week (Ohhira *et al.,* 1990). The perfect *Belacan* with a pleasant flavor and aroma is produced after one or two weeks of fermentation. A high quality *Belacan* should consist of about 50% moisture content and 13-17% salt (Adnan, 1984). *Belacan* with low moisture content is very dry and hard. Traditionally, *Belacan* is used as a flavor enhancer in several Malaysian dishes. It is also mixed with chillies and lime as a dipping condiment known as *sambal-belacan* which is much favored by Malaysians (Hutton, 2013). *Belacan* has strong umami taste and contain considerable amounts of glutamate and 5'-nucleotides glutamate content (Khairunnisak *et al.,* 2009; Jinap *et al.,* 2010). The concentration of free glutamic acid found in different brands of Malaysian *Belacan* was reported to be 180-530 mg/100 g. The total concentration of 5'-nucleotides of belacan ranges from 0.85 to 42.25 mg/g

(Jinap *et al.,* 2010). Jinap *et al.* (2010) reported up to 32-fold increase in umami attributes when *Belacan* was added to Malaysian dishes. *Belacan* has been identified as one of the major umami contributors in Malaysian cuisines. Dishes containing *Belacan* have been shown to have high intensity of meaty flavor, which represent umami taste (Jinap *et al.,* 2010).

Terasi, a fermented shrimp paste, is a traditional condiment used commonly in Indonesia. It is produced from the planktonous shrimp *Schizopodes* or *Mytis* sp. Shrimp paste (*Terasi-udang*) is much more favored than fish paste (*Terasi-ikan*) for most Indonesians. *Terasi-udang* is usually mixed with chilli, garlic and salt, which they call it *sambal-terasi*. *Terasi-udang* is made from shrimp mixed with approximately 15% salt. The mixture is fermented (usually takes 6-9 months) until the desired *Terasi* aroma is developed. In Java Island, shrimp paste is often prepared from pre-cooked shrimps called *Brabon* (literally means mother paste). *Terasi* is used as a flavor enhancer in various Indonesian dishes. *Terasi-udang* is dark brown, grey or red paste in color with a distinct taste and strong aroma (Kobayashi *et al.,* 2003). It has considerable level of free glutamic acid (1508.56 mg/100 g) and is rich in umami taste (Mizutani *et al.,* 1987).

Petis-udang is also another local shrimp paste widely consumed in Indonesia. The shrimp wastes (heads and shells) are boiled to produce *Petis-udang*. The waste is by-product of Indonesian shrimp crackers (*krupuk-udang*) processing, in which the shrimp meat is used. *Petis-udang* is used as a seasoning in various Indonesian-style salad dressings and other dishes (Sobhi *et al.,* 2013). The nucleotide content in *Petis-udang* is not very high, but it contains a variety of free amino acids, such as glycine, alanine, and glutamic acid (Jinap *et al.,* 2010).

In Myanmar, shrimp paste is called *Seinsanga-pi* or *Hmyinnga-pi.* It is a pink to reddish color paste made from very small *Mysis* or planktonic shrimps. The paste made from quality shrimp is called *Seinsanga-pi* and the rest are basically called *Hmyinnga-pi*. To produce this type of shrimp paste, shrimp is mixed with salt and dried under the sun for 3 to 4 days. The mass is later pounded with the addition of extra salt and water to obtain paste. The paste is sun dried again for a day and pounded for 3-4 times to get a homogeneous and smooth paste. The paste is then fermented for 4 to 6

month. This shrimp paste is rich in umami taste. *Hmyinnga-pi* contains considerable amounts of free glutamic acid (10.08 mg/100 g) (Tyn, 1983).

Salty fermented shrimp paste is called *Bagoong-alamang* in the Philippine. *Bagoong-alamang* is consumed raw or cooked and is generally used as flavoring or condiment in many traditional Filipino dishes. The characteristics of this product vary among different parts of the Philippines. In the Tagalog provinces, the paste is completely fermented and ground, with or without the addition of coloring matter. In the Ilocos and Pangasinan provinces, it is either partially or completely fermented. In the Visayas and Mindanao provinces, the product is slightly fermented without any liquid (Olympia, 1992). *Bagoong-alamang* is normally fermented for 10 days. According to Peralta *et al.* (2008), the content of *L*-glutamic acid in *Bagoong-alamang* increased from 25.8 to 38.2 mg/100 g during the 10 days of fermentation. The increase in free glutamic acid during fermentation enhances the desirable umami taste in the product. Prolonged fermentation of shrimp paste for more than one year brings about a decrease in free amino acids, especially free glutamic acid. The shrimp paste made in the Philippines was reported to contain higher free glutamic acid (814.15 mg/100 g) as compared to those made in other Southeast Asia countries (Mizutani *et al.,* 1987).

In Thailand, *Jaloo* is an indigenous salt fermented shrimp (*Macrobrachium orientalis* or *Macrobrachium lanchesteri*) produced by the residents of the coastal areas in the south of Thailand. *Jaloo* is prepared in the same manner as *Kapi* except that the drying is not required. Fermentation of *Jaloo* under anaerobic conditions generally takes 2-3 days (Faithong *et al.,* 2010). *Koong-som* is another fermented shrimp product of Thailand produced by mixing small shrimp (*Acetes* sp.) with salt and palm-sap-sugar concentrate as a source of carbohydrate. The mixture is usually fermented by lactic acid bacteria for the development of a sour taste and the typical flavor of fermented shrimp (Faithong *et al.,* 2010).

Fermented shrimp products are usually high in umami taste components. For instance, *Belacan* contains more than 4,200 mg free glutamic acid/100 g. *Terasi* and *Kapi* are also rich in free glutamic acid which is responsible for the umami taste in the product. Levels of free glutamic acid (mg/100 g) in fermented shrimp products from different countries are shown in Table 4.

Table 4. Levels of free glutamic acid (mg/100 g) in fermented shrimp products from different countries

Country	Fermented shrimp products	Free glutamic acid (mg/100 g)
Malaysia	*Belacan*	4207
	Cencaluk	854
Indonesia	*Terasi*	1508
Thailand	*Kapi*	1647
Philippines	*Bagoong-alamang*	814
Vietnam	*Nuoc mam*	740
Burma	*Hmyin nga pi*	10
	Ngan pya ye	54.16

Source: modified from Hajeb and Jinap (2013).

1.2.3.2 Raw material of fermented shrimp products

Shrimps of the genera *Acetes, Mesopodopsis, Lucifer* and *Mysids* are usually used to produce fermented shrimp products (Hajeb and Jinap, 2013). However, shrimp species used are depending on shrimp available in each country. The genus *Acetes* is the most common raw materials to produce shrimp sauce, paste and other fermented products. Table 5 shows that *Acetes* species, mostly used to produce fermented shrimp products in Southeast Asian countries. Among them, *A. indicus, A. erythraeus, A. vulgaris* and *A. japonicus* are the most commonly used species (Ruddle and Ishige, 2010).

Table 5. Shrimp of *Acetes* species used for fermentation in Southeast Asian countries

Country	Species
Burma	*A. indicus*, *A. intermediuc*, *A. vulgaris*
Indonesia	*A. japonicas*, *A. sibogaesibogae*
Malaysia	*A. japonicas*, *A. erythraeus*, *A. sibogaesibogae*
Philippine	*A. erythraeus*, *A. intermedius*, *A. vulgaris*
Singapore	*A. erythraeus*, *A. indicus*, *A. vulgaris*
Thailand	*A. japonicas*, *A. vulgaris*

Source: Ruddle and Ishige (20010).

For *Kapi* production, salted shrimp paste of Thailand, planktonous krill (*Mesopodopsis orientalis*) is typically used as the traditional raw material of this

product. Since 1990, krill stocks have dropped by 3% per year (Meland and Willassen 2007). Small shrimp belonging to genus *Acetes* have been recently used as alternative raw material to produce *Kapi*.

1.2.3.3 Microorganisms of fermented shrimp products

Microflora plays an important role in the preparation of fermented shrimp pastes. The presence of microorganisms during fermentation contributes to the degradation of proteins and the development of flavor and aroma. The microbial contents of various fermented shrimp pastes show broad diversity ranging from LAB belonging to coccal-lactics (*Lactococcus, Enterococcus*) to species of homofermentative and heterofermentative rods (*Lactobacillus*), endospore-forming rods (*Bacillus*), aerobic coccus (*Micrococcus*), to species of yeast (*Candida*, *Saccharomycopsis*), as well as pathogenic contaminants such as *B. cereus*, *S. aureus* and *Enterobacteriaceae* (Sand and Crisan, 1974). The other predominant microorganism present in fermented shrimp paste was *Bacillus* spp. The presence of *Bacillus* spp. is due to their ability to form endospores to survive under prevailing conditions (Sand and Crisan, 1974). Previously, several microbiological studies of shrimp paste have been conducted. The microflora of fish or shrimp paste, *Bagoong*, in the Philippines include *Bacillus* sp., *Micrococcus* sp., and *Moraxella* sp. (Beddows and Ardeshir, 1979). *Belacan* microflora include *Bacillus, Pediococcus, Lactobacillus, Corynebacteria, Micrococcus, Clostridium, Brevibacterium* and *Flavobacterium.* The predominant microorganisms are LAB, Micrococcus, *Bacillus* sp. and high salt tolerant species (Sobhi *et al.,* 2013). Surono and Hosono (1994) reported that the microflora of *Terasi,* which consisted of 11 species of *Bacillus*, 4 species of *Pse*udomonas, 3 species of *Micrococcus*, one species of *Kurthia* and one species of *Sporolactobacillus*. *Terasi,* the fermented shrimp of Indonesia, is made by the slow digestion or fermentation of salted shrimp and the subsequent separation of the solid from the liquid portion of the hydrolysate (Kobayashi *et al.,* 2003). The endogenous enzymes from *Bacillus subtilis* and *B. coagulans* in addition to those found in the intestines caused protein hydrolysis. The bacterial enzymes were mainly responsible for deamination and decarboxylation of amino acids. Lower MW fatty acids and amides formed also produce the characteristic flavor of the product. *B. pumilus* is the dominant species throughout the

fermentation process. Other bacteria responsible for early-stage fermentation were *B. coagulans, B. megaterium* and *B. subtilis,* while later stage fermentation was dominated by *B. licheniformis*, *Micrococcus colpogenes*, *M. roseus*, *M. varians* and *Staphylococcus* species (Kobayashi *et al.,* 2003). Chotwanawirach (1980) also documented that some strains of lactic acid bacteria including *Staphylococcus*, *Micrococcus* and *Bacillus* were detected in *Kapi.* Moreover, several halophilic bacteria with protease activity have been reported in *Kapi.* Those included *Lentibacillus kapialis, Salinicoccus siamensis, Virgibacillus halodenitrificans* and *Oceanobacillus kapialis* (Pakdeeto *et al.,* 2007a, b; Namwong *et al.,* 2009; Tanasupawat *et al.,* 2011).

Normally, most of fermented shrimp pastes contain high salt (25-30%). Thus microorganisms found during production are generally classified as halophile. The important roles of bacteria in fermented food are protein degradation and flavor-aroma development (Disarapong, 2005). Consequently, when it is produced under aseptic conditions, typical aroma or volatile compounds is not developed (Beddows and Ardeshir, 1979). Bacteria involved in general fermented food can be classified into two major groups: (1) bacteria that produce proteolytic enzymes and (2) bacteria that contribute to flavor and aroma development.

1.2.3.4 Physical and chemical composition of fermented shrimp products

Fermented shrimp paste is basically protein hydrolysates that result from the natural fermentation of shrimp and salt kept in tanks/earthenware jar at an ambient temperature for 1-12 months (Saisithi, 1994). Similar with fish sauce, fermented shrimp paste mostly contains high amount of salt and the nutrient intake from this product is expected to be quite limited (Beddows and Ardeshir, 1979). The proximate and chemical compositions of some fermented shrimp paste products are shown in Table 6.

Table 6. pH and proximate composition of fermented shrimp paste products

Compositions (%)	*Kapi* (Thai shrimp paste)	*Saewoojeot* (Korean shrimp paste)	*Belacan* (Malaysian shrimp paste)	*Terasi* (Indonesian shrimp paste)	*Bagoong-alamong* (Philippine shrimp paste)
pH	7.44-7.66	8.50	7.56	6.9-7.5	7.75
Moisture	36.78-49.93	21.13	47.92	30-50	33.08
Ash	24.13-28.99	13.95	19.15	10-40	43.90
Protein	20.14-25.12	56.59	30.38	20-40	14.79
Fat	1.53-2.22	0.82	0.63	2-5	0.91
Carbohydrate	2.30-15.61	NM	NM	3.5-5	NM
Salt content	19.29-24.73	15.22	14.94	23-30	24.42
Reference	Faithong *et al.* (2010)	Kim *et al.* (2014)	Karim (1993)	Roling and Van-Verseveld (1996)	Montano *et al.* (2001)

NM, Not mentioned

Fermented shrimp pastes also contain nutritional properties similar to fish sauces. The amino acid compositions of shrimp paste are quite comparable to fish sauce. In addition, the polyunsaturated fatty acids (PUFAs), present in the final product, i.e., 20:5n-3 (EPA) and 22:6 n-3 (DHA), can survive oxidation during the 360 day fermentation (Montano *et al.,* 2001). This is due to the presence of antioxidants in the shrimp paste. Free amino acids (FAA) have been reported to be able to prevent oxidation. Maillard reaction products (MRPs) have been identified as the antioxidants present in shrimp paste (Peralta *et al.,* 2008). Tsai *et al.* (2006) studied some characteristics of commercial fermented fish products in Taiwan, including fish sauce, fish paste and shrimp paste. The levels of pH, salt content, total volatile basic nitrogen, trimethylamine, and aerobic plate count in all samples were in the ranges of 4.8-6.5, 16.2-45.3%, 51-75 mg/100 g, 5.4-53.9 mg/100 g and 1.0-4.2 log CFU/g, respectively. The average content for each sample of all different biogenic amines was less than 90 ppm, except for histamine which has an average content of 394 ppm in fish sauce, 263 ppm in fish paste, and 382 ppm in shrimp paste. Most of the tested fermented fish products (92.6%) had histamine levels greater than the FDA guideline of 50 ppm, while seven of them (25.9%) contained >500 ppm of histamine. Additionally, *Bacillus coagulans* and *Bacillus megaterium* were identified as the two histamine-producing bacteria capable of producing 13.7 and 8.1 ppm of histamine in fish sauce, respectively.

Flavor is one of the most important factors governing fermented shrimp pastes quality. Several volatile components of this product associated with its flavor have been reported. More than 150 volatile compounds have been identified in fish and shrimp pastes (Cha *et al.,* 1998). The compounds consist of aldehydes, ketones, alcohols, aromatic compounds, *N*-containing compounds, esters, *S*-containing compounds and some other compounds. The aldehyde content is high in all fish pastes and low in shrimp paste. Among the aldehydes, benzaldehyde is found in all pastes. Benzaldehyde is reported to possess a pleasant almond, nutty and fruity aroma (Vejaphan *et al.,* 1988). Ketones are more abundant in all fish pastes and are much less in shrimp paste. Among the ketones found in fish pastes (including shrimp paste), 2,3-butanedione, has been reported to contribute an intense buttery and desirable aroma in crustaceans (Tanchotikul and Hsieh, 1989). Alcohols are found in abundance in all fish pastes, but are low in shrimp pastes. The low content of alcohol in shrimp pastes correspond to their low aldehyde content. Among the phenolic or aromatic compounds, toluene is more abundant in shrimp pastes, while phenol is more abundant in fish pastes. Toluene and phenol have been reported to give an undesirable aroma in seafood (Vejaphan *et al.,* 1988). Shrimp pastes contain more *N*-containing compound as compared to fish pastes. Among these *N*-containing compounds, 2-ethyl-3,6 dimethylpyrazine is found in big-eye herring, hair-tail viscera and shrimp pastes. This compound has been reported to contribute to nutty, roasted and toasted aromas. Pyrazines are formed through the Maillard reaction of amino acids. Various esters have also been identified in fish pastes. Ethylbutanoate provides a cheesy flavor to anchovy and big-eye herring paste. Four major *S*-containing compounds found in fish and shrimp pastes are dimethydisulfate, dimethyltrisulfate, isobutylisopropylsulfate, and 3,5-dimethyl-1,2,4-trithiolate. The presence of these *S*-containing compounds may affect the overall flavor because of their low thresholds. Furans are present in fish and shrimp pastes. Furans have been reported to have burnt, sweet, bitter and coconut-like flavor in some foods (Maga and Katz, 1979). High content of protein was observed in *ngari*, *hentak* and *tungtap* (Thapa *et al.,* 2004). *Tungtap* has high calcium (5040 mg/100 g) and phosphorus (1930 mg/100 g) contents among the minerals (Agrahar-Murugkar and Subbulakshmi, 2006).

Kapi, salted shrimp paste of Thailand, has been produced from various types of shrimp or krills. Chemical characteristics of two types of Thai traditional fermented shrimp pastes, *Kapi Ta Dam* and *Kapi Ta Deang,* were comparatively studied by Kleekayai *et al.* (2014). *Kapi* products had 13-17% (w/v) salt content since salt was added for controlling spoilage and proliferation of pathogenic microorganisms. Water activity (A_w) of *Kapi Ta Dam* and *Kapi Ta Deang* were 0.63 and 0.66, with the pH of 7.2 and 8.0, respectively. Both samples showed similar amino acid composition. Lysine and leucine were predominant essential amino acids in both products, while the major non-essential amino acids were glutamic acid and aspartic acid. A high amount of those non-essential amino acids including glutamic acid, aspartic acid, and glycine, was reported as a taste attribute in fish and shellfish products (Jung *et al.,* 2004). *Kapi* collected from the southern part of Thailand had a moisture content in the range of 36.78-49.93% and protein contents of 20.14-25.12%. Fat contents of *Kapi* were from 1.53% to 2.22%. Moreover, a wide range of carbohydrate content (2.30-13.05%) was noticeable. All *Kapi* samples contained a high salt content, ranging from 19.91% to 24.73%. The pH of all samples were in the range of 7.44-7.66 as reported by Faithong *et al.* (2010). Chemical composition of 14 samples of shrimp paste from local Klongkone producers were analyzed by Prapasuwannakul and Suwannahong (2015). Samples contained 37.36-46.85% moisture, 20.95-30.86% ash, 18.95-25.14% protein, 0.69-2.05% lipid, 4.27-17.96% carbohydrate and 19.78-22.96% salt. Water activity of the samples were in the range of 0.70-0.74. Chemical and physical properties of 13 *Kapi* samples were also examined by Daroonpunt *et al.* (2016). The moisture content and water activity (A_w) of the products ranged from 33.95-52.19% and 0.64-0.72, respectively, while salt concentration was from 7.00 to 10.85%. The nitrogen content varied from 2.87 to 6.85% (w/w). The predominant amino acids were glutamic acid (70.1-593.9 μg/g), lysine (112.7-546.3 μg/g) and leucine (29.5-544.9 μg/g). Total bacterial cell count in the samples ranged from 1.3×10^3- 2.9×10^5 CFU/g, while lactic acid bacteria (LAB) were not detected. Therefore, the compositions and characteristics found in *Kapi* samples varied with raw material, ingredients and processes used.

1.2.3.5 Antioxidative properties or bioactivities of fermented shrimp products

Protein components in food also include bioactive peptides, which could exert a physiological effect in the body. These short chain peptides containing amino acids are inactive within the sequence of the parent protein, but can be released by either microorganisms or endogenous proteases during gastrointestinal digestion, food processing or fermentation (Wijesekara and Kim, 2010). Peptides usually contain 2-20 amino acid residues per molecule, but may consist of more than 20 amino acids. Following digestion, bioactive peptides can either be absorbed through the intestine to enter the blood circulation intact and exert systemic effects, or produce local effects in the gastrointestinal tract. Depending on the amino sequence, these peptides can exhibit diverse activities (Erdmann *et al.,* 2006). Bioactive peptide exhibit various regulatory effects such as antioxidant (Je *et al.,* 2005), immune defense, opioid and antihypertensive activities (Yu *et al.,* 2006). Fishery-derived bioactive peptides have been obtained widely by enzymatic hydrolysis of fish proteins. In fermented fishery products such as fish sauce and blue mussel sauce, enzymatic hydrolysis was carried out by microorganisms and bioactive peptides were present in the products (Ichimura *et al.,* 2003; Jung *et al.,* 2006). Fishery-derived bioactive peptides have been shown to possess antioxidative activity and ACE inhibition.

Antioxidative activity has been found in a number of fermented fishery products such as fermented blue mussels (Jung *et al.,* 2006), fish sauces (Harada *et al.,* 2007; Michihata *et al.,* 2002) and fermented shrimp paste (Peralta *et al.,* 2008). The antioxidative activity is attributed to certain amino acid sequences (Erdmann *et al.,* 2006). Peptides with the molecular weight of approximately 1400 Da and with 5-16 amino acid sequences showed strong inhibition on the auto-oxidation of linoleic acid (Wu *et al.,* 2003). Several amino acids, such as tyrosine, methionine, histidine, lysine and tryptophan, were generally accepted as antioxidants (Harada *et al.,* 2007). High amounts of histidine and some hydrophobic amino acids are related to the antioxidant potency (Pena-Ramos *et al.,* 2004). The activity of histidine-containing peptides is thought to be connected to hydrogen-donating ability, lipid peroxyradical trapping and/or the metal ion chelating ability of the imidazole group (Zhu *et al.,* 2004). The

addition of a leucine or proline residue to the *N*-terminus of a histidine-histidine dipeptide would enhance antioxidative activity (Lertprakobkit, 2011). According to Chen *et al.* (1996), histidine and proline play an important role in the antioxidative activity of peptides from soy bean hydrolysate. Pro-His-His showed the greatest antioxidative activity among all tested peptides and had synergistic effects with nonpeptidic antioxidants. The hydrophobicity of the peptide also appears to be an important factor for its antioxidative activity due to increased accessibility to hydrophobic targets (e.g., lipophilic fatty acids) (Chen *et al.,* 1996). Moreover, protein hydrolysates and peptides with an increase in hydrophobicity would increase their solubility in lipid and therefore enhances their antioxidative activity (Rajapakse *et al.,* 2005; Zhu *et al.,* 2004).

Fermented fishery products are good source of peptide and amino acid, and become the rich sources of structurally diverse bioactive compounds. Thus, fishery proteins are of particular interest especially promising due to their high protein content and diverse physiological activities in the human organism (Wu *et al.,* 2003). Antioxidative activities of enzymatically hydrolyzed shrimp products, shrimp waste and fermented shrimp products have been previously reported. Binsan *et al.* (2008) reported antioxidative activity in *Mungoong*, an extract paste from the cephalothorax of white shrimp (*Litopenaeus vannamei*), towards $ABTS^{+}$ and DPPH radicals as well as FRAP. Distilled water was the best potential solvent extracting antioxidant compounds from *Mungoong* among all solvents tested. Moreover, Peralta *et al.* (2008) found that the antioxidative activities of 80% ethanol extract derived from the Philippine salt-fermented shrimp paste against DPPH radical, hydrogen peroxide and lipid peroxidation were increased through the prolonged fermentation time. Faithong *et al.* (2010) reported antioxidative activity of Thai traditional fermented shrimp and krill products including *Jaloo, Koong-Som* and *Kapi* against DPPH and $ABTS^{+}$ radicals and FRAP. A water-soluble fraction from *Kapi* showed the strongest antioxidative activity amongst soluble fractions from all products. Kleekayai *et al.* (2014) documented that the water extracts from *Kapi Ta Dam* (*Mesopodopsis orientalis*) and *Kapi Ta Deang* (*Acetes* spp.) showed strong antioxidative activities, especially against $ABTS^{+}$ radical as well as ACE inhibitory activity. Nevertheless, the extracts did not exhibit antimicrobial activity against *Staphylococcus aureus, Bacillus cereus, Escherichia coli*

and *Salmonella Typhimurium*. Biological activities in *Kapi* could be developed by fermentation process, enzymatic hydrolysis of proteins and non-enzymatic browning reactions. In addition, a cholesterol lowering effect of salted and fermented small shrimp (*Acetes japonicas*) extract was also observed in high cholesterol-diet induced hypercholesterolemic animal models (Seok *et al.,* 2004). *Kapi* could therefore serve as a potential source of natural bioactive substances.

1.2.4 Fish and shellfish proteases

According to the International Union of Applied Biochemists classification, proteases from fish and aquatic invertebrates can be classified into four major groups (Simpson, 2000).

1.2.4.1 Acid/Aspartyl proteases

Aspartyl proteases have been described as a group of endopeptidase characterized by high activity and stability at acid pH (Whitaker, 1994). They are referred to as “aspartyl” proteinases (or carboxyl proteinases) because their catalytic sites are composed of the carboxyl group of two aspartic acid residues (Simpson, 2000). Based on the EC system, all aspartyl proteases from marine animals have the first three digits in common: EC 3.4.23. Three types of aspartyl proteases that have been isolated and characterized from the stomachs of marine animals are pepsin, chymosin and gastricsin (Simpson, 2000). Pepsin is assigned the number EC. 3.4.23.1. It has preferential specificity for the aromatic amino acids, phenylalanine, tyrosine, and tryptophan. In the EC system of classification, chymosin (formerly known as rennin) is assigned the number EC 3.4.23.4. Chymosin has specificity for the aromatic amino acids, phenylalanine, tyrosine, and tryptophan, similar to pepsin. Gastricsin is assigned a code of EC 3.4.23.3. Pepsin is composed of a single polypeptide chain of 321 amino acids and has a molecular weight (MW) of 35 kDa (Simpson, 2000). Many previous studies found that pepsins and pepsin-like enzymes can be extracted from the digestive glands of marine animals such as Atlantic cod (*Gadus morhua*) (Brewer *et al.,* 1984), Montery sardine (*Sardinops sagax caerulea*) (Castillo-Yañez *et al.,* 2004) or salmon fish (*Oncorhynchus keta*) (Sánchez-Chiang *et al.,* 1987), etc. Acid proteinases were

found in the extract from Antarctic krill (*Euphausia superba*) and the two major acid proteinases, A and B, were purified 80-140 fold. The molecular weights of acid proteinases A and B were estimated by gel filtration on Sephadex G-100 to be approximately 45 and 64 kDa, respectively. The optimal pH of acid proteinases A and B was 3.0 toward hemoglobin. They were partially inhibited by acid proteinase specific reagents including diazoacethyl-*DL*-norleucine methylester (DAN) and 1,2epoxy-3-(*p*-nitrophenoxy) propane (EPNP). Cathepsin D is the major lysosomal pepstatin-sensitive aspartic protease which participates in intracellular breakdown of tissue proteins. It may also be involved in post-mortem tenderness of fish muscle caused by fragmentation of myofibrils (Jiang *et al.,* 1992). Cathepsin D were characterized from the shrimp muscle, for example, Pacific whiting (*Merluccius productus*) (Erickson *et al.,* 1983), the banded shrimp (*Penaeus japonicas*) and grass shrimp (*Penaeus monodon*) (Jiang *et al.,* 1992), etc.

1.2.4.2 Serine proteases

The serine proteases are a group of endopeptidase with a serine residue together with an imidazole group and an aspartyl carboxyl group in their catalytic sites (Simpson, 2000). The proteases in serine subclass have the same first three digits on the EC system as EC 3.4.21. Serine proteases exhibit high activity under alkaline rather than neutral pH and sensitivity to serine protease inhibitors. The three major serine proteinases purified and well characterized from the marine animals are trypsin, chymotrypsin and elastase. Trypsin is assigned the code ED 3.4.21.4. Trypsin has a very narrow specificity for the peptide bonds on the carboxyl side of arginine and lysine. Generally, serine protease, especially trypsin, is considered to be the major proteolytic enzyme in many crustaceans (Sriket *et al.,* 2011). Trypsins have been isolated and characterized from various shrimp or krill species. Trypsin from digestive gland of the shrimp *Penaeus japonicus* was also purified. It had the MW of 25 kDa with the optimal pH and temperature of 8 to 8.3 and 60°C, respectively (Galgani *et al.,* 1985). Sriket *et al.* (2012) reported that the highest proteolytic activity of purified trypsin from the hepatopancreas of freshwater prawn (*Macrobrachium rosenbergii*) was found at pH 7 and 60°C when casein was used as a substrate. It had a molecular weight of 17 kDa

and stable to heat treatment up to 40°C. It was stable over a pH range of 7.0-11.0. Furthermore, trypsin activity was strongly inhibited by soybean trypsin inhibitor, *N-ρ*-tosyl-*L*-lysine chloromethyl ketone (TLCK) and was partially inhibited by ethylenediaminetetraacetic acid (EDTA). Wu *et al.* (2008) purified trypsin from North Pacific krill (*Euphausia pacifica*) by ammonium sulfate precipitation and ion-exchange and gel-filtration chromatography. MW of trypsin was 33 kDa. It was active over a wide pH (6.0-11.0) and temperature (10-70°C) range. It had the optimum pH of 9.0 and optimal temperature of 40-50°C. Trypsin was stable between pH 6.0 and 11.0 and below 30°C. Trypsin from Antarctic krill processing wastewater, purified using affinity chromatography on *p*-aminobenzamidine Sepharose 4B, had MW between 32 and 33 kDa. The optimal pH and temperature were pH 8 and 60°C, respectively (Bustos *et al.,* 1999). Sun *et al.* (2014) studied the autolysis and characterized the protease extract of North Pacific krill (*Euphausia pacifica*). Three major proteinases with approximate MW of 26 kDa (KP-1), 18 kDa (KP-2) and 17 kDa (KP-3) were found. KP-3 was active over a pH range from pH 5.0-12.0. The highest autolytic activity was observed at pH of 7 and temperature of 60°C. The proteolytic activity of KP-1, KP-2 and KP-3 toward casein was strongly suppressed by Pefabloc SC, serine proteinase inhibitors. Senphan *et al.* (2015) documented that proteases from hepatopancreas of Pacific white shrimp (*Litopenaeus vannamei*) mainly consisted of trypsin. This trypsin, purified using ammonium sulfate precipitation and a series of chromatographies including diethylaminoethyl sepharose and soybean trypsin inhibitor sepharose 4B columns, had a molecular weight of 24 kDa. The optimal pH and temperature were 8.0 and 60°C, respectively. It was stable to heat treatment up to 60°C and over a pH range of 7.0-11.0. The activity was strongly inhibited by *N-ρ*-tosyl-*L*-lysine chloromethyl ketone.

Chymotrypsin is assigned a code of EC 3.4.21.1 and it has a much broader specificity than trypsin. It cleaves peptide bonds involving amino acids with bulky side chains and nonpolar amino acids such as tyrosine, phenylalanine, tryptophan, and leucine. Chymotrypsin has been also isolated and characterized from various shrimp and krill species. Tsai *et al.* (1986) purified chymotrypsins from shrimp (*Penaeus monodon*) by DEAE-cellulose ion exchanger at pH 7.0 and gel filtration chromatography. It was found to be single-chained with MW of 27 and 26 kDa. They

were effectively inhibited by soybean trypsin inhibitor, turkey and chicken ovomucoid, chymostatin, PMSF and Z-AlaGlyPhe chloromethyl ketone. Hernandez-Cortes *et al.* (1997) purified chymotrypsin from hepatopancreas of the white shrimp, *Penaeus vannamei,* by Q-Sepharose fast flow and Phenyl-Q-sepharose column chromatography. MW of chymotrypsin was 33.2 kDa. It had the optimum pH of 8.0 when SAAPFpNA was used a substrate. Shi *et al.* (2008) isolated chymotrypsin from the hepatopancreas of Chinese shrimp (*Fenneropenaeus chinensis*). MW of the purified chymotrypsin by CNBr-activated Sepharose 4B was approximately 25 kDa. And it was inhibited by PMSF, TPCK and SBTI. Chymotrypsin-like enzyme of Antarctic krill (*Euphausia superba*) was characterized with respect to purity by the means of capillary electrophoresis and matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF-MS). The molecular weight of the chymotrypsin-like proteinases was 26,260 Da. The highest proteolytic activity of this enzyme was found at 55°C and pH 8 (Sjodahl *et al.,* 2002). Navarrete-del-Toro *et al.* (2015) purified a chymotrypsin from the digestive gland of shrimp (*Penaeus californiensis*) using an affinity chromatography, soybean trypsin inhibitor-agarose gel. This enzyme had MW of 35.7 kDa. Compared to bovine chymotrypsin, shrimp chymotrypsin was more stable at higher temperatures (50°C), sensitive to low pH, possessed an acidic pI and was activated by calcium.

1.2.4.3 Thiol/Cysteine proteases

Thiol or cysteine proteinases are a group of endoproteases that have cysteine and histidine residues as the essential groups in their catalytic sites. These enzymes require the thiol (-SH) group furnished by the active site cysteine residue to be intact, hence this group is named “thiol” or “cysteine” proteinases (Whitaker, 1994). The thiol proteinases are inhibited by heavy metal ions and their derivatives, as well as by alkylating agents and oxidizing agents (Whitaker, 1994). The first three digits common to thiol proteinases are EC 3.4.22 (Simpson, 2000). Digestive cysteine or thiol proteases have been found in digestive organ of marine animals, which are most active at acidic pH and inactive at alkaline pH (Simpson, 2000). Common examples of digestive thiol protease from marine animals are cathepsin B, cathepsin L and cathepsin S (Simpson, 2000). Cathepsin was isolated and characterized from some shrimp species

such as cathepsin L from shrimp (*Metapenaeus ensis*) (Hu and Leung, 2004), cathepsin B from white shrimp (*Litopenaeus vannamei*) (Stephens *et al.,* 2012) and cathepsin C from black tiger shrimp (*Penaeus monodon*) (Qui *et al.,* 2008).

1.2.4.4 Metalloproteases

The metalloproteases are hydrolytic enzymes whose activity depends on the presence of bound divalent cations (Simpson, 2000). Chemical modification studies suggest that there may be at least one tyrosyl residue and one imidazole residue associated with the catalytic sites of metalloproteinases (Whitaker, 1994). The metalloproteases have a common first three digits as EC 3.4.24 (Simpson, 2000). The metalloproteases are inhibited by chelating agents such as 1,10-phenanthroline, EDTA, and sometimes by the simple process of dialysis (Whitaker, 1994). The metalloproteses have been characterized from marine animals (e.g., rockfish, carp, and squid mantle) but have not been found in the digestive glands except in the muscle tissue (Simpson, 2000). Metalloproteases do not seem to be common in marine animals (Simpson, 2000). However, metalloprotease (gelatinase) was isolated from hepatopancreas of the marine crab (Sivakumar *et al.,* 1999). The enzyme had a MW of 55 kDa and was active against native type I collagen. Optimum temperature and pH were 25°C and 7-7.5, respectively. Sivakumar *et al.* (1999) reported that this enzyme was strongly inactivated by 10 mM EDTA.

1.2.5 Pigments (astaxanthin and carotenoid)

Astaxanthin is the predominant carotenoid in penaeids. Three forms of astaxanthin, namely diester, monoester, and free, were found in black tiger shrimp (Okada *et al.,* 2009). The main carotenoid in the carotenoprotein was free astaxanthin, and no significant difference was found between astaxanthin diester and astaxanthin monoester (Okada *et al.,* 2009). This complex can be green, purple, or blue in the living animal, acquiring a red color when subjected to heat treatment (Britton, 1996). Yanar *et al.* (1997) reported that caroteinoid contents of shrimps (*Penaeus semisulcatus* and *Metapenaeus monoceros*) varied with seasons. Generally the complexes of carotenoids and proteins (carotenoproteins and carotenolipoproteins) were prevalent (Britton,

1996). The red color of cooked crustaceans appears by the release of the individual astaxanthin prosthetic groups from the carotenoproteins when denatured by heat (Okada *et al.,* 2009).

Dietary supplementation of astaxanthin or its precursors improved or corrected the color of penaeids, especially those intensively cultured, for better market price (Yuan *et al.,* 2011). In nature, protein-pigment interaction increases carotenoid stability (Britton, 1996). The use of proteolytic enzymes to disrupt the protein-pigment bond, increased carotenoid extraction by 58% or spitted the chitin-pigment interaction, resulting in protein enriched pigments (Hansen *et al.,* 2000).

Some studies have demonstrated that astaxanthin may possess strong antioxidative activity and exert a protective effect against chronic diseases such as cancer (Ikeda *et al.,* 2008). Antioxidative activity of astaxanthin was higher than β-carotene and α-tocopherol (Britton, 1996; Ikeda *et al.,* 2008). However, astaxanthin is susceptible to isomerization and oxidative degradation, because of the presence of long-chain conjugated double bonds (Yuan *et al.,* 2011). The formation of cis-isomers of astaxanthin or its derivatives may possess different biological activity. The carotenoid content in the wastes from Indian shrimps was found to vary from 35 to 153 μg/g depending on the species and the major pigments were astaxanthin and its esters (Sachindra *et al.,* 2005).

1.2.6 Change during fermentation of fishery and meat products

During fermentation, microbiological and biochemical change occur. Proteolysis is an essential biochemical reaction occurring during the fermentation of fermented products. It is induced by endogenous proteases in shrimp as well as those produced by halophilic bacteria surviving under high salt condition (Gildberg and Stenberg, 2001). Proteolysis affects both texture and flavor of fermented fish products by inducing the formation of low molecular weight compounds, e.g. peptides, amino acids, aldehydes, organic acids and amines (Mizutani *et al.,* 1987). Lipolysis during fermentation is also important since free fatty acids (FFA) released undergo oxidation responsible of aroma development (Lizaso *et al.,* 1999). Oxidation products including aldehydes and ketones (Takeungwongtrakul and Benjakul, 2013) are also generated during fermentation step. Overall, during fermentation of fish or shrimp pastes, proteins

and fat as well as glucose undergo degradation to produce amino acids and peptides, fatty acids, organic acids and other non-protein nitrogen compounds. These compounds more likely contribute to the development of typical taste and flavor of fermented product (Lizaso *et al.,* 1999).

1.2.6.1 Chemical and biological changes

1.2.6.1.1 Changes in pH

Due to different protein hydrolysis, the difference in pH probably resulted from the differences in free hydrogen ions, free amino acids, and amino acid of oligopeptides (Gildberg and Stenberg, 2001). The decrease in pH might be associated with organic acids, such as lactic acid, acetic acid produced by some microorganisms, especially lactic acid bacteria during fermentation (Ijong and Ohta, 1996). Conversely, the increase in pH might be caused by the formation of basic compounds (Gildberg and Stenberg, 2001). Lopetcharat *et al.* (2001) reported that the pH of Pacific whiting fish sauce fermented for 40 days ranged from 6.1 to 6.3. Ijong and Ohta (1996) reported that pH of *Bakasang* (Indonesian fermented fish sauce) ranged from 5.95 to 6.50, whereas Aquerreta *et al.* (2002) found a pH range of 4.90-5.42 in *Garum* (Greece fermented fish sauce). The pH of *Garum* increased during the fermentation process, probably as a consequence of the accumulation of basic compounds. Shih *et al.* (2003) found that the increase in pH was probably due to the increase of total volatile bases (TVB) during the fermentation.

1.2.6.1.2 Changes in salt content

Salt content in fermented food is normally constant, especially with increasing fermentation time. Yongsawatdigul *et al.* (2007) reported that salt content of fish sauce prepared from Indian anchovy (*Stolephorus indicus*) was approximately 25-26%. Lopetcharat *et al.* (2001) reported that salt content in the fish sauce from Pacific whiting waste increased at day 5. Thereafter, it remained constant at about 25-30% during 60 days of fermentation. Salt concentration reached equilibrium with increasing fermentation time (Chayovan *et al.,* 1983). Chin and Koehler (1983) reported that the

very high concentration of salt used in the mixture during fermentation might inhibit the growth of microorganisms that could decarboxylate free histidine to histamine.

1.2.6.1.3 Changes in nitrogenous compounds

The soluble nitrogen components including proteins, peptides, and amino acids are generated from fish or shellfish fermentation by the activities of proteolytic enzyme. Nitrogenous compounds in fish sauce are composed of protein and nonprotein nitrogenous (NPN) compounds such as free amino acid, nucleotides, peptide, ammonia, urea and TMAO (Thiansilakul *et al.,* 2007). Total nitrogen content is used as an indicator to determine the grade and price of fish sauce or even fermented food in Thailand. In fish sauce, products containing total nitrogen content over 20 g/l are classified as Grade I and 15 to 20 g/l as Grade II (Thai Industrial Standard, 1983). Total nitrogen contents from Pacific whiting fish sauce increased from 6.40 to 15.7 g N/l during the first 10 day of fermentation (Lopetcharat *et al.,* 2001). Tungkawachara *et al.* (2003) reported that total nitrogen content in fish sauce increased during fermentation. At the early stage of fermentation (1 and 3 month), fish sauce produced from Pacific whiting mixed with by-product (WB) had higher total nitrogen content than fish sauce produced from Pacific whiting (W), possibly due to the greater degree of hydrolysis. W and WB fish sauce had total nitrogen contents of 1.28 and 1.36%, respectively. Brillantes and Samosorn (2001) reported that total nitrogen content of fish sauce produced from fresh anchovy ranged from 3.0 to 4.9 g/l. It increased dramatically during the fermentation period especially in the first 4 months, then remained relatively constant after 6 month. Fish sauce produced from low quality fish contained the high total nitrogen levels (4 to 9 g/l) in the first-week of fermentation, increased significantly until 7 months and then remained relatively constant with final value of 18 to 22 g/l at the end of 12 months. Siringan *et al.* (2006) found that total nitrogen content of fish sauce prepared from Indian anchovy (*Stolephorus indicus*) increased to reach the plateau of 2.1 to 2.3 g N/100 ml within 25 weeks and remained relatively constant until 52 weeks.

The increase in formal nitrogen content suggested the increased hydrolysis of peptides (Tungkawachara *et al.,* 2003) caused by the endogenous or microbial proteinases. Not only free amino acid or peptide but also ammonia was

produced during the extended fermentation time. Ammonia might be formed by deamination process (Lopetcharat *et al.,* 2001). Lopetcharat *et al.* (2001) reported that the ammonia nitrogen reached a maximum level at day 10 and then decreased to a minimum at day 30. The increased ammonia nitrogen content during the first 15 day could be due to fish enzymes that were active during early stage of fermentation (Beddows and Ardeshir, 1979). However, the diminishing of ammonia in the fish sauce might be caused by the slow dissipation into the air. Another tentative explanation is the formation of Schiff base in the reaction of amine with aldehyde or ketone group (Tungkawachara *et al.,* 2003).

Amino nitrogen is usually used as an indicator for degree of fermentation (Lopetcharat *et al.,* 2001). The amino nitrogen content represents the amount of amino groups in the product. An increase in amino nitrogen concentration is related to the degradation of the polypeptide (Tungkawachara *et al.,* 2003). The longer fermentation time allowed more breakdown of soluble protein and peptide into free amino acids and volatile nitrogen (Chaveesuk *et al.,* 1994). The increased amino nitrogen contents could be due to hydrolytic activity of fish protease, which were active during the early storage of fermentation (Beddows and Ardeshir, 1979). Tungkawachara *et al.* (2003) reported that amino nitrogen content were about 50 to 52% of the total nitrogen in Pacific whiting fish sauce after 3 months of fermentation.

1.2.6.1.4 Changes in bioactivity

During fermentation, peptides are generated. Additionally, some chemical reaction related with color development takes place. Peralta *et al.* (2008) studied the antioxidative activity and nutritional components of Philippine salt-fermented shrimp paste. Antioxidative activity was improved through prolonged fermentation (90, 180, and 360 days). The activity was suggested to be related with the Maillard reaction products formed. Polyunsaturated fatty acids like eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) in the shrimp paste were not substantially damaged for 360 days, while free amino acid content dramatically increased at 90 days. However, an excessive fermentation showed significant decrease in free amino acids and increase in ammonia. The decrease in amino acids indicates further degradation of amino acids to produce amines, volatile acids and other nitrogenous substances.

Properly prolonged fermentation would improve antioxidant ability and some nutritional value in the salt-fermented shrimp paste (Peralta *et al.,* 2008).

1.2.6.2 Physical changes

Color is another quality attribute, which can be altered in fermented fish during the fermentation process. The Maillard reaction bring about a variety of intermediate products and finally brown pigments (melanoidins) are formed during fermentation (Van Boekel, 2006). Lertsiri *et al.* (1993) reported that free amino acids and reducing sugar generated during fish sauce fermentation contributed to the development of brown pigment via Maillard reaction. Fluorescent compounds are possible precursors of brown pigments (Van Boekel, 2006). Most of the nitrogenous compounds in fermented food, especially fish sauce, are free amino acids and small peptides, which contribute to brown color development. Even though reducing sugar content in fish is low, carbohydrate derivatives, such as glucose-6-phosphate and other substances present in the metabolic pathways, can also act as reactants to initiate the Maillard reaction (Yongsawatdigul *et al.,* 2007). Kim *et al.* (2004) found that the color L^* value of salted and fermented anchovy sauce decreased during storage.

1.2.6.3 Sensorial changes

The effects of ingredients and processing, enzymatic and chemical processes, which occur during fermentation and storage, have a considerable influence on the organoleptic quality of fermented meat products. Breakdown products of lipolysis and proteolysis i.e. amino acids, carbonyls and volatile flavor compounds contribute to the characteristic flavor of fermented meats (Phithakpol, 1993). Aroma is an important indicator of the quality of fermented food such as fish sauce, soy sauce, etc (Shih *et al.,* 2003). Nitrogenous compounds have been known to contribute to the flavor and nutritional values of fermented products. The volatile acids were the most abundant volatile compounds in fermented seafood (Sanceda *et al.,* 2001). In general, fish spoilage increased the amount of volatile acids during fish sauce fermentation. Volatile acid contents in the aerobically fermented fish sauce were significantly higher than in the anaerobically fermented fish sauce (Sanceda *et al.,* 2001). The differences

in the aroma of fish sauce were thought to be due to the differences in the level of concentrations of the major acids (Shih *et al.,* 2003). Differences in the aroma of the commonly used fish sauce from different countries were attributed to the aroma characteristics of the major as well as the minor volatile compounds (Shih *et al.,* 2003). Degradation of fish protein to free amino acid is primarily responsible for the delicious taste of fermented food (Chayovan *et al.,* 1983). Various volatile compounds, including acids, carbonyls, nitrogen-containing compounds and sulfur-containing compounds, are formed during fermentation and believed to be responsible for the distinct aroma of fermented food (Sanceda *et al.,* 2001).

Fermented seafood such as fish sauce, shrimp paste, oyster sauce produced from different raw materials and processes might possess different sensory characteristics. Tungkawachara *et al.* (2003) reported no significant difference in overall sensory acceptance and flavor acceptance for all fish sauce produced from Pacific whiting (*Merluccius productus*) and Pacific whiting mixed with surimi by-product. However, fish sauce made from Pacific whiting mixed with surimi by-product has lower color acceptance scores than commercial anchovy fish sauce. No significant difference in color acceptance was detected between Pacific whiting fish sauce and commercial anchovy fish sauce. Chaveesuk *et al.* (1994) found no difference in the color, aroma and flavor scores between the commercial fish sauce and that produced from Atlantic herring (*Clupea harengus*) with enzyme supplement. Nevertheless, commercial fish sauce had a slightly stronger flavor and aroma than the enzyme supplemented fish sauce.

1.2.6.4 Microbiological changes

Microorganisms play an essential role during fermentation process. Thongthai and Siriwongpairat (1990) demonstrated the presence of at least two bacterial populations in fish sauce from anchovy. The major population consisted of red extremely halophilic bacteria, identified as halobacteria. These reached maximum density in the liquor after 3 weeks and persisted throughout the fermentation period, whereas the other minor bacterial population was heterogenous and halotolerant or moderately halophilic.

In fish sauce, microbial counts decreased continuously when the fermentation time increased, possibly caused by high concentrations of salt and reduced pH. Lopetcharat *et al.* (2001) reported that an increase in the number of halophilic microorganisms was observed at day 10 during Pacific whiting fish sauce fermentation and then decreased rapidly to an undetectable level at day 20. Three significant microorganism genera were identified in fish sauce from Pacific whiting including *Staphyloccus, Bacillus,* and *Micrococcus* (Lopetcharat *et al.,* 2001). *Bacillus* sp. and *Staphyloccus* strain 109 were isolated from fish sauce and produced a measurable amount of volatile acids (Ijong and Otha 1996). Furthermore, *Staphyloccus, Micrococcus* and *Coryneform* are commonly found in anchovy fish sauce (Sand and Crisan, 1974). The microorganism should play an important role in the later stage of fermentation and the ripening stage. Protein degradation by these microorganism leads to the production of volatile compounds from amino acids and small peptides (Lopetcharat *et al.,* 2001). Fukuchi *et al.* (2003) reported that bacterium, which was isolated from fish sauce mush (*moromi*) of frigate mackerel and identified as *Staphylococcus xylosus*, could change notes of an odor in fish sauce made in Thailand. Volatile compounds of the fish sauce after incubation at 32°C for 24 days with the cultured bacterium were produced. *Bacillus, Micrococcus, Streptococcus, Pediococcus*, and other halophilic bacteria that produce lactic acid are found in fish sauce, including *Nam-pla, Shotturu, Bakasang* and *Nouc-mam* (Tanasupawat and Komagata, 1995; Ijong and Ohta, 1996). However, it is unclear how these bacteria act on the production of the characteristic taste and odor of fish sauce during fermentation.

DGGE has been used to monitor microbial dynamics during production of many fermented products. This technique has been developed and is being widely used in the study of the microbial flora of fermented foods such as fermented sausages, fermented grains, fermented meat and fermented dairy products (Fujii *et al.,* 2011). DGGE technique was used to monitor the dynamic changes in bacterial community during the ripening of natural fermented Italian sausages by using DNA and RNA directly extracted from the food matrix as described by Cocolin *et al.* (2004). The region V1 of the 16S rDNA was taken into account for the analysis of the meat samples while PCR and RT-PCR were used to obtain amplicons to be separated by DGGE. Lactic acid bacteria were found in the early stage of the ripening along with other organisms,

mainly members of the family *Micrococcaceae* and meat contaminants, such as *Brochothrix thermosphacta* and *Enterococcus* spp. LAB represented the main population, especially *Lactobacillus sake* as well as *L. curvatus* and were responsible for the acidification and proteolysis that determined the organoleptic features of the fermented sausages, while *Micrococcaceae* were shown to have a restricted importance compared to LAB.

Yoshigawa *et al.* (2010) elucidated the microbial dynamics of fermented chum salmon sauce (FCSS) inoculated with various combinations of halotolerant microorganisms (HTM) starters and barley *koji*, and clarify interferences among starter and other microorganisms by the plate count method and PCR-DGGE method. Nine different combinations of *mugi koji* (barley steamed and molded with *Aspergillus oryzae*) and HTMs (*Zygosaccharomyces rouxii*, *Tetragenococcus halophilus* and *Candida versatilis*) were inoculated into chum salmon sauce mash under a non-aseptic condition used in industrial fish sauce production and fermented at 35±2.5°C for 84 days to elucidate the microbial dynamics (i.e., microbial count and microbiota) during fermentation. The viable count of halotolerant yeast (HTY) in fermented chum salmon sauce (FCSS) mash showed various time courses dependent on the combination of the starter microorganisms. Halotolerant lactic acid bacteria (HTL) were detected morphologically and physiologically only from FCSS mash inoculated with *T. halophilus* alone or with *T. halophilus* and *C. versatilis* during the first 28 days of fermentation. Only four fungal species, *Z. rouxii, C. versatilis, Pichia guilliermondii,* and *A. oryzae* were detected throughout the fermentation by PCR-DGGE.

1.2.7 Starter culture for fermented fishery and meat products

The use of starter cultures in meat/seafood fermentations has become well established recently as a means to increase processing rates and product consistency. Additionally, many research reported that starter culture can improve the sensory characteristics and microbiological quality of meat product (Casaburi *et al.,* 2008). Microorganisms are well known for excretion of proteolytic enzymes capable of degrading proteins. Many types of microbes excrete proteolytic enzymes, including the fungi *Aspergillus oryzae*, the bacteria *Bacillus subtilis*, the actinomycetes *Streptomyces griseus*, and the yeast *Saccharomyces* spp. (Mackie *et al.,* 1982). Careful selection by

seeding or controlling the growth environment within the fermentation chamber enables the desired microbes to flourish and produce significant quantities of proteolytic enzymes which help hydrolyze the fish protein (Casaburi *et al.,* 2008).

Protein hydrolysis during fermentation is normally accomplished by the action of both endogenous proteinases and bacterial proteinases (Saisithi *et al.,* 1994). Activity of endogenous proteinases decreased at high salt environment, resulting in slow rate of protein hydrolysis. Halophilic bacterial proteinase showing activity and stability at high salt content could be a potential source to accelerate the fermentation process of fermented food (Thongthai *et al.,* 1992). Chaiyanan *et al.* (1999) reported that halophilic and halotolerant proteinase-producing bacteria including *Halobacterium salinarium* and *H. thailandensis* sp., were isolated from fish sauce fermentation. These strains showed caseinolytic and gelatinolytic activity. Proteinases from *Bacillus licheniformis, B. amyloliquefaciens* and *Bacillus subtilis* had the decreased activity under high NaCl content. Activity of *B. subtilis* JM-3 proteinase decreased with NaCl concentration (Kim and Kim, 2005), while proteinase activity from *Filobacillus* sp. RF2-5 increased 2.5 times in the presence of 15-25% NaCl (Hiraga, 2005). Therefore, to successfully accelerate protein hydrolysis at high salt content, proteases exhibiting high stability and activity at 25-30% NaCl should be sought and characterized (Sinsuwan *et al.,* 2008).

Lipolysis, together with proteolysis, is believed to play a central role in aroma formation. This phenomenon is only the first step in the process and is followed by further oxidative degradation of fatty acids into alkanes, alkenes, alcohols, aldehydes and ketones (Casaburi *et al.,* 2008) that enhances the development of the flavor. In fact, medium and long-chain fatty acids act as precursors of aroma compounds whereas the short-chain fatty acids ($C<6$) lead to strong cheesy odors (Ansorena *et al.,* 2002). Although tissue lipases are primarily responsible for lipolysis during the fermentation, numerous studies over the last decade described lipolytic bacteria, especially *Staphylococci* sp. (Benito *et al.,* 2007) played an important role in lipolysis. Moreover, Martuscelli *et al.* (2000) described the capability of *Staphylococcus xylosus* and *Staphylococcus carnosus* strains to modulate the aroma through the conversion of amino acids and free fatty acids (FFA). Strains of *S. xylosus* were recommended for the production of very aromatic sausages of southern Europe (Benito *et al.,* 2007).

Therefore, the use of well-selected strains with lipolytic and/or proteolytic activity, could generate high amounts of aroma components, thereby achieving improved sensory quality (Casaburi *et al.,* 2008).

LAB starters (*Pediococcus acidilactici*) were used in *Som-fug*, a Thai fermented fish mince, to reduce fermentation time and improved it quality. *Som-fug* inoculated with *P. acidilactici* at 10^4 CFU/g (PA104) had a greater acceptability than those inoculated with *Lactobacillus plantarum* and *Pediococcus pentosaceus* at either 10^4 or 10^6 CFU/g and the control (without inoculum). During fermentation, *P. acidilactici* exhibited a higher rate of fermentation than the control as indicated by the greater rate of pH drop and lactic acid production. Additionally, inoculated *Som-fug* generally exhibited higher hardness and adhesiveness than the control (Riebroy *et al.,* 2004).

The effect of starter cultures on proteolytic activity and amino acid content in fermented sausages was investigated (Aro-Aro *et al.,* 2010). Five types of commercial starter cultures were selected. Sausages inoculated with *Lactobacillus sakei* and *Staphylococcus carnosus* had the higher proteolytic activity than those inoculated with *Pediococcus pentosaceus* and *Staphylococcus xylosus.* The proteolytic activity of both inoculated sausage was higher than the control. Moreover, a slight increase in proteolytic activity was detected during storage in both sausages. The marked differences in the content of free amino acids at the end of the process could be attributed to the starter culture activity.

Casaburi *et al.* (2008) studied the effect of proteolytic and lipolytic starter cultures on traditional fermented sausage ripening and sensory properties. *Lactobacillus curvatus* and *Staphylococcus xylosus* strains, with lipolytic and proteolytic activities, were isolated and inoculated. Higher proteolysis and lipolysis were observed in sausages inoculated with proteolytic and lipolytic *S. xylosus* coupled with *L. curvatus*, while the sausage inoculated with only *S. xylosus* without *lactobacilli* was identical to the non-inoculated control, indicating that the proteolysis could be due to both microbial activity and endogenous proteases activated by the decrease in pH. The sausages ripened without starter addition and those started without the *L. curvatus* AVL3 showed similar sensory features.

Effect of starter cultures on microbiological and physico-chemical characteristics of *Suan yu,* a traditional Chinese low salt fermented fish was studied by Zeng *et al.* (2013). Three groups of mixed starter cultures (S1: *Lactobacillus plantarum* 120*, Staphylococcus xylosus* 135 and *Saccharomyces cerevisiae* 31; S2: *L. plantarum* 145, *S. xylosus* 135 and *S. cerevisiae* 22; S3: *Pediococcus pentosaceus* 220; *S. xylosus* 135 and *S. cerevisiae* 22), were inoculated to produce the traditional fermented fish. After 42 days of fermentation at 24°C, *Suan yu* inoculated with different mixed starter cultures had the rapid growth of lactic acid bacteria (LAB), declined pH and suppressed increase of thiobarbituric acid (TBARS) and total volatile base nitrogen (TVB-N) as well as growth of spoilage bacteria and pathogens. Besides, *Suan yu* had higher contents of non-protein nitrogen (NPN) and total free amino acids (FAA) compared to the control. The fermented fish was more widely accepted than the control. Therefore, the inoculation with S1, S2 and S3 reduced the lag time of fermentation and improved the quality of *Suan yu.*

The microbiological, physico-chemical and chemical properties of dry fermented mutton sausages inoculated with starter culture (combination of *Lactobacillus pentosus, Pediococcus pentosaceus* and *S. carnosu*) were different from the control (without inoculum) during ripening and storage. Lactic acid bacteria populations increased significantly to log 9 CFU/g and were higher than the control (log 8 CFU/g) during fermentation and ripening. The total free fatty acid (TFFA) in the inoculated sausage increased significantly during ripening and storage (Zhao *et al.,* 2011).

Virgibacillus sp. SK33, newly isolated from one-month-old Thai fish sauce, was studied for its proteinase production. Extracellular proteinase of *Virgibacillus* sp. SK33 exhibited optimum activity at 50°C and pH 8, 10 and 11. Crude proteinase was strongly inhibited by phenylmethanesulfonyl fluoride (PMSF). All proteinases exhibited caseinolytic activity at 25% NaCl. Proteolytic activity towards synthetic substrate increased about 4 times in the presence of 25% NaCl, indicating the characteristic of NaCl-activated proteinase. In addition, crude proteinase from *Virgibacillus* sp. SK33 showed higher proteolytic activity towards anchovy than did commercial proteinases at 25% NaCl, demonstrating its potential for protein hydrolysis under high salt condition (Sinsuwan *et al.,* 2008).

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1.4 Objectives

1.4.1 To study physical, chemical compositions and some properties of commercial *Kapi,* collected from various regions of Thailand.

1.4.2 To characterize the endogenous proteases in *A. vulgaris* and *M. lanchesteri* and to monitor the changes in proteolytic activity during *Kapi* fermentation.

1.4.3 To investigate the impact of post-mortem storage time of shrimp, *A. vulgaris* and *M. lanchesteri*, prior to salting on quality of resulting *Kapi.*

1.4.4 To comparatively examine compositions/properties as well as quality of *Kapi* produced from shrimp *A. vulgaris* and *M. lanchesteri*

1.4.5 To monitor biochemical and microbiological changes of shrimp *A. vulgaris* during *Kapi* production

1.4.6 To characterize lipids from shrimp, *A. vulgaris* and to monitor their changes during *Kapi* production.

1.4.7 To study the growth and production of extracellular enzymes from *Bacillus* spp. K-C3, isolated from commercial *Kapi* and to evaluate the quality of *Kapi* inoculated with this *Bacillus* strain, compared with control (without inoculum).

CHAPTER 2

CHEMICAL COMPOSITION AND PHYSICAL PROPERTIES OF SALTED SHRIMP PASTE (*KAPI*) PRODUCED IN THAILAND

2.1 Abstract

Chemical composition and physical properties of 11 salted shrimp pastes (*Kapi*) obtained from various places of Thailand were determined. Based on proximate composition, protein constituted the major component (29.44-53.27%, dry weight basis). All samples contained 22.77-35.47% NaCl with A_w of 0.695-0.774. Various formal nitrogen contents (11.96-22.87 mg N/g sample) were in agreement with different degrees of hydrolysis (12.68-20.76%), suggesting the varying cleavage of peptides among the samples. From electrophoretic study, salted shrimp paste contained a large amount of small molecular weight proteins and peptides. Different samples had different colors with ΔE^* of 47.10-60.43 and ΔC^* of 9.46-20.76. The samples had total carotenoid content of 0.54-1.97 mg/g sample. Free astaxanthin, astaxanthin diester and canthaxanthin were the major carotenoids in salted shrimp paste. Thus, salted shrimp paste is a good source of protein and serves as the nutritious condiment.

2.2 Introduction

Fermented fishery products are extensively consumed in Southeast Asian countries since the fifteenth century, and consumed as staples, side dishes or condiments/seasonings in daily foods. The widespread consumption of fishery fermented products over a wide geographical area throughout Southeast Asia is due to the simplicity of the processing techniques and uniformity of the final fermented products (Hajeb and Jinap, 2013). Those products impart delicacy and have high nutritional value (Faithong *et al.,* 2010).

During fermentation, raw materials are converted into products through activities of endogenous enzymes or microorganisms (bacteria, yeasts and molds). The process can be a natural process, in which desirable microorganisms grow

preferentially, or a controlled process, in which isolated and characterized fermentative microorganisms (starter culture) are added to the raw material under controlled conditions (Tamang and Kailasapathy, 2010). Fermentation of fishery products does not play a role in preserving them, and smaller peptides and amino acids generated can serve as nutrients for as microorganisms. Therefore, fermentation is often combined with the addition of salt or drying to reduce water activity and eliminate proteolytic and putrefying microorganisms (Chaveesak *et al.,* 1993).

Fermentation process is also influenced by several variables including (1) the microflora present in the raw material and salt, (2) proteolytic activity of raw material and microorganism, (3) condition of the product entering the fermentation process, (4) presence or absence of oxygen, (5) nutritional state of the raw material, (6) temperature, (7) pH of the fermentation mixture, (8) the presence of visceral enzymes, (9) the presence and concentration of carbohydrates, and (10) the duration of the fermentation process (Phithakpol and Kasetsat, 1995). Although some products have similar process, some ingredients used can be varied, leading to the different characteristics, especially flavor and taste (Mizutani *et al.,* 1987).

Kapi, a typical traditional salted shrimp paste, has been widely consumed in Thailand as a condiment. Salted shrimp paste is generally prepared from the planktonous shrimp or krill (*Acetes vulgaris* or *Mesopodopsis orientalis*). Traditionally, salted shrimp paste is produced by mixing one part of salt with three to five parts of shrimp or krill. The mixture is salted, pounded and spread out on the ground to dry under sunlight. The paste is compacted and allowed to ferment in anaerobic condition at ambient temperature (25–35°C) for at least 1 month or longer (Faithong *et al.,* 2010). The color of salted shrimp paste varies from a pinkish or purplish gray to a dark grayish brown. The consistency also varies, from soft and pasty to dry and hard. It could be preserved for several months (Phithakpol, 1993). However, raw material, shrimp or krill/salt ratio, fermentation process and time can be varied, depending on regions or countries. This leads to different characteristics and properties of the product (Peralta *et al.,* 2008). However, a little information regarding the chemical compositions and physical properties of salted shrimp paste (*Kapi*) produced in different places in Thailand has been reported.

2.3 Objective

To determine the chemical composition and physical properties of salted shrimp paste (*Kapi*) collected from various regions of Thailand.

2.4 Materials and methods

2.4.1 Chemicals

Sodium sulfite and ammonium thiocyanate were obtained from Riedel–de Haen (Seelze, Germany). Potassium persulfate, acrylamide, bis-acrylamide and *N,N,N',N'*-tetramethyl-ethylenediamine (TEMED) were procured from Fluka Chemical Co. (Buchs, Switzerland). Anhydrous sodium sulfate was obtained from Merck (Darmstadt, Germany). Isopropanol, petroleum ether and hexane were purchased from Lab-Scan (Bangkok, Thailand). Astaxanthin and b-carotene were obtained from Dr. Ehrenstorfer GmbH (Augsburg, Germany).

2.4.2 Sample collection

Kapi samples were purchased from different provinces in Thailand, including Sa-tun (1 sample) [S1], Ranong (2 samples) [S2, S3], Krabi (2 samples) [S4, S5], Songkhla (2 samples) [S6, S7], Samut Sakhon (1 sample) [S8], Rayong (1 sample) [S9], Chachoengsao (1 sample) [S10] and Samut Songkram (1 sample) [S11]. Those *Kapi* were produced from different raw material including 1) krill (*Mesopodopsis* spp.): S4, S5, S8, S9, S11; 2) shrimp (*Acetes* spp.): S1, S2, S3, S6, S7; and 3) shrimp (*Mysis* spp.): S10. Each sample was divided into several portions (100 g each), placed in polyethylene bag and heat-sealed. The samples were kept at -20°C and the storage time was not longer than 2 months.

2.4.3 Proximate analysis

Moisture, ash, fat, protein and carbohydrate contents of *Kapi* were determined according to AOAC methods (2000) with the analytical No. of 35.1.13, 35.1.14, 35.1.25, 35.1.15 and 35.1.16, respectively.

2.4.4 Determination of salt content

Salt content of *Kapi* was determined according to AOAC method (2000). Sample (0.5–1 g) was mixed with 10–20 ml of 0.1 N $AgNO_3$ and 10 ml of Conc. HNO_3. The mixture was boiled on a hot plate for 10 min and then cooled under running water. The mixture was filtered through a filter paper (Whatman No. 1) (Whatman International Ltd., Maidstone, UK). The filtrate was adjusted to the volume of 50 ml by distilled water and 5 ml of ferric alum indicator was added. The mixture was titrated with standardized 0.1 N KSCN until the solution became permanent brownish-red. The salt content was then calculated and expressed as %NaCl using the following equation:

$$\text{NaCl (\%)} = \frac{[(\text{ml of } AgNO_3 \times \text{conc. of } AgNO_3)\text{-}(\text{ml of KSCN} \times \text{conc. of KSCN}] \times 5.85}{\text{Weight of sample (g)}}$$

2.4.5 Water activity (A_w) and pH determination

The A_w of *Kapi* was measured using a water activity analyzer (Thermoconstanter, Novasina, Switzerland). The pH of samples was measured according to the method of Nirmal and Benjakul (2009) with a slight modification. *Kapi* (3 g) was homogenized thoroughly with 10 volumes of distilled water (w/v). The homogenate was kept at room temperature for 5 min prior to measurement using a pH meter (Sartorius, Gottingen, Germany).

2.4.6 Determination of formal nitrogen, ammonia nitrogen and amino nitrogen contents

2.4.6.1 Formal nitrogen content

Formal nitrogen content was determined by the titration method as described by Thai Industrial Standard (1983) with a slight modification. *Kapi* (2 g) was mixed with 10 ml of distilled water. Then, the mixture was homogenized at a speed of 9,500 rpm for 2 min using an IKA Labortechnik homogenizer (Selangor, Malaysia). Ten ml of formalin solution (38%, v/v; pH 9) were added and mixed well. The mixture was titrated with 0.1 N NaOH to obtain pH of 9.0. Formal nitrogen content was calculated and expressed as mg formal nitrogen/g sample using the following equation:

$$\text{Formal nitrogen content (mg N/g)} = \frac{\text{ml of NaOH (pH 7 - pH 9)} \times 0.1 \times 14}{\text{Weight of sample (g)}}$$

2.4.6.2 Ammonia nitrogen content

Ammonia nitrogen content was determined by the titration method as described by Thai Industrial Standard (1983) with a slight modification. *Kapi* (2 g) was placed in 400 ml Kjeldahl flask containing 100 ml of distilled water and 3 g of MgO. The mixture was distilled and the distillate (approximately 8-10 ml) was collected in 50 ml of 4 % (w/v) boric acid containing the mixed indicators (0.125 g methyl red and 0.082 g bromocresol green in 95% alcohol (100 ml): 0.1% methylene blue in distilled water with ratio of 5:1). The solution was then titrated with 0.05 N H_2SO_4 to reach the end point. Ammonia nitrogen content was calculated as follows:

$$\text{Ammonia nitrogen content (mg N/g)} = \frac{5.6 \times 0.05 \times \text{ml of } H_2SO_4}{\text{Weight of sample (g)}}$$

2.4.6.3 Amino nitrogen content

Amino nitrogen content was calculated based on the difference between formal and ammonia nitrogen contents as described by Thai Industrial Standard 1983.

2.4.7 Measurement of degree of hydrolysis (DH)

DH of *Kapi* was determined according to the method of Benjakul and Morrissey (1997). The sample (1 g) was mixed with 9 ml of 5% (w/v) SDS. The mixture was homogenized at a speed of 11,000 rpm, 1 min. The homogenate was heated at 85°C for 30 min. The mixture was then subjected to centrifugation at 10,000×*g* for 15 min at room temperature (Model RC-B Plus centrifuge Newtown, CT, USA). Thereafter, 2.0 ml of 0.2 M phosphate buffer (pH 8.2) and 1.0 ml of 0.01% (w/v) TNBS solution were added. The solution was mixed thoroughly and placed in a temperature-controlled water bath (Memmert, Schwabach, Germany) at 50°C for 30 min in the dark. The reaction was terminated by adding 2.0 ml of 0.1 M sodium sulfite. The mixture was then cooled at room temperature for 15 min. The absorbance was read at 420 nm and free amino group content was expressed in terms of *L*-leucine. DH was calculated as follows:

$$\text{Degree of hydrolysis (\%)} = \frac{L}{L_{max}} \times 100$$

where L is the amount of free amino group in the product and L_{max} is the total free amino group after acid hydrolysis (6 M HCl at 100°C for 24 h).

2.4.8 Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE)

Protein patterns were determined by SDS-PAGE using 4% stacking gel and 12.5% running gel, according to the method of Laemmli (1970). Samples (3 g) were solubilized in 27 ml of 5% (w/v) SDS (85°C). The mixture was homogenized for 1 min at a speed of 13,000 rpm and incubated at 85°C for 1 h to dissolve total proteins. Samples (15 μg protein) determined by the Biuret method (Robinson and Hogden, 1940) were loaded onto the gel and subjected to electrophoresis at a constant current of 15 mA per gel using a Mini-Protean II unit (Bio-Rad Laboratories, Inc., Richmond, CA, USA). After electrophoresis, the gels were stained with 0.05% (w/v) Coomassie Blue R-250 in 50% (v/v) methanol and 7.5% (v/v) acetic acid for 30 min. Finally, gels were destained with a mixture of 50% (v/v) methanol and 7.5% (v/v) acetic acid for 30 min and destained again with a mixture of 5% (v/v) methanol and 7.5% (v/v) acetic acid for 1 h.

2.4.9 Analysis of carotenoid

Total carotenoid content in the samples was determined according to the method of Saito and Regier (1971) with a slight modification. Sample (30 mg) was mixed with 10 ml of petroleum ether and homogenized at a speed of 10,000 rpm for 1 min. The homogenate was allowed to stand for 30 min, followed by filtration using a Whatman filter paper No.4 (Whatman International Ltd., Maidstone, UK). The absorbance of the filtrate, appropriately diluted, was read at 468 nm using a UV-1601 spectrophotometer (Shimadzu, Kyoto, Japan). The concentration (C) of carotenoid in the sample was calculated as following equation:

$$C\ (\text{mg/g lipids}) = \frac{A_{468} \times \text{Vol. of extract} \times \text{dilution factor}}{0.2 \times \text{weight of lipids (g)}}$$

where 0.2 is the A_{468} of 1 µg/ml standard astaxanthin.

The filtrate containing carotenoid was subjected to thin-layer chromatography (TLC) using activated 20×20 cm silica gel plates (silica gel G type 60, Merck) following the modified procedure described by Sanchez-Camargo *et al.* (2011). The sample was applied on the plates, and the separation was carried out using a mixture of isopropanol and hexane (50:50, v/v) as mobile phase. β-carotene and astaxanthin were used as standards.

2.4.10 Measurement of color

Color of *Kapi* was determined using a colorimeter (ColourFlex, Hunter Lab Reston, VA, USA) and reported in the CIE system. L^* (lightness), a^* (redness/greenness), and b^* (yellowness/blueness) were reported. Additionally, ΔE^* (total difference of color) and ΔC^* (the difference in chroma) were calculated as follows:

$$\Delta E^* = \sqrt{(\Delta L^*)^2 + (\Delta a^*)^2 + (\Delta b^*)^2}$$

where ΔL^*, Δa^* and Δb^* are the differentials between color parameters of the samples and those of the white standard (L^* = 93.55, a^* = -0.84 and b^* = 0.37).

$$\Delta C^* = C^*_{sample} - C^*_{standard}$$

where $C^* = \sqrt{(a^*)^2 + (b^*)^2}$

2.4.11 Statistical analysis

All analyses were conducted in triplicate. Statistical analysis was performed using one-way analysis of variance (ANOVA). Mean comparison was carried out using Duncan's multiple range test (Steel *et al.,* 1980).

2.5 Results and discussion

2.5.1 Proximate compositions, Aw, pH and salt content

Proximate compositions of different *Kapi* samples are shown in Table 7. Moisture content of *Kapi* varied from 33.79 to 52.54%. Based on dry matter, protein constituted the major component (29.44-53.17%), indicating that *Kapi* could be the good source of proteins. S11 (*Kapi* Samut Songkram) showed the highest protein content (53.17%) followed by S9 (*Kapi* Rayong) (48.19%). All samples contained salt, which is needed for preservation. Salt content was in the range of 22.77-35.47%. This contributed to high ash content of all samples (33.80-50.50%). S6 (*Kapi* Songkhla1) and S7 (*Kapi* Songkhla2) had the highest salt contents (35.47 and 34.44%, respectively). This was in agreement with the highest ash contents (50.08 and 50.50%, respectively). Slightly higher ash content, in comparison with salt content, was more likely attributed to the presence of inorganic substances in the shell of shrimp or krill used as raw material. Shell has been known to have $CaCO_3$ as the component (Stevenson, 1985). Fat contents of salted shrimp paste ranged from 1.41 to 3.67%. Shrimp oil was reported to be rich in polyunsaturated fatty acid. Linoleic acid (C18:2(n-6)) was the dominant fatty acid in shrimp oil from Pacific white shrimp (Takeungwongtrakul *et al.,* 2012b). This made shrimp oil susceptible to lipid oxidation (Takeungwongtrakul and Benjakul, 2013). Moreover, a wide range of carbohydrate content (4.90-32.48%) was noticeable. S4 (*Kapi* Krabi1) had the highest carbohydrate content (32.48%). S1 (*Kapi* Satun) and S10 (*Kapi* Chachoengsao) also contained a high amount of carbohydrate (20.99 and 22.75%, respectively). The difference in carbohydrate content could be obtained among the samples, dependent on the addition of polysaccharides by processors. They add the root crops, flour or sugar to increase the yield or improve the flavor and taste of shrimp paste (Faithong *et al.,* 2010). Those components had the impact on quality attribute and nutritive value of salted shrimp paste. Faithong *et al.* (2010) reported that pH, moisture, protein, fat, ash, salt and carbohydrate content of *Kapi* produced in the south of Thailand were 7.44-7.66, 36.78-49.93, 31.85-43.12, 2.42-4.10, 41.04-50.93, 19.29-24.73 and 4.58-24.70% (dry weight basis), respectively.

Table 7. Proximate compositions, salt content, A_w and pH of various commercial *Kapi*

Samples*	Moisture (%)	Ash (%)	Protein (%)	Fat (%)	Carbohydrate (%)	Salt (%)	A_w	pH
S1	35.36±0.81^{f}	24.11±0.08^{e} (37.30±0.13)^{H}	24.07±0.34^{f} (37.23±0.52)^{I}	1.76±0.07^{a} (2.72±0.11)^{B}	14.7±0.53^{b} (22.75±0.61)^{B}	19.76±0.21^{d} (30.57±0.30)^{D}	0.669±0.00^{g}	7.94±0.01^{f}
S2	47.65±0.18^{b}	24.46±0.04^{d} (46.73±0.07)^{C}	21.86±0..09^{g} (41.76±0.16)^{G}	1.33±0.15^{bc} (2.54±0.28)^{BC}	4.7±0.12^{g} (8.97±0.38)^{G}	19.65±0.03^{d} (30.41±0.05)^{D}	0.732±0.00^{b}	8.14±0.13^{b}
S3	47.06±0.11^{c}	23.59±0.06^{f} (44.57±0.12)^{D}	19.31±0.06^{i} (36.47±0.13)^{J}	0.75±0.2^{d} (1.41±0.39)^{F}	9.29±0.33^{d} (17.55±0.64)^{D}	18.26±0.08^{e} (28.25±0.13)^{E}	0.727±0.00^{c}	8.4±0.07^{a}
S4	40.25±0.23^{d}	21.47±0.05^{h} (35.93±0.08)^{I}	17.59±0.15^{j} (29.44±0.24)^{K}	1.28±0.05^{bc} (2.14±0.09)^{DE}	19.41±0.42^{a} (32.48±0.40)^{A}	16.71±0.04^{g} (25.84±0.05)^{G}	0.708±0.00^{f}	7.99±0.11^{d}
S5	47.75±0.12^{b}	20.53±0.24^{i} (39.29±0.45)^{F}	24.69±010^{e} (47.26±0.18)^{C}	1.28±0.10^{bc} (2.46±0.20)^{BCD}	5.75±0.19^{f} (10.99±0.32)^{E}	17.23±0.100^{f} (26.65±015)^{F}	0.734±0.00^{b}	8.03±0.12^{c}
S6	40.73±0.07^{d}	29.68±0.14^{a} (50.08±0.24)^{B}	25.23±0.07^{d} (42.57±0.12)^{F}	1.45±0.06^{b} (2.45±0.10)^{BCD}	2.91±0.18^{h} (4.90±0.28)^{J}	22.93±0.15^{a} (35.47±0.23)^{A}	0.712±0.00^{e}	7.97±0.03^{e}
S7	47.11±0.04^{c}	26.71±0.02^{b} (50.50±0.03)^{A}	20.64±0.07^{h} (39.03±0.13)^{H}	1.13±0.12^{c} (2.14±0.22)^{DE}	4.41±0.09^{g} (8.33±0.29)^{H}	22.26±0.25^{b} (34.44±0.39)^{B}	0.72±0.00^{d}	7.81±0.03^{g}
S8	37.81±0.07^{e}	25.91±0.01^{c} (41.66±0.01)^{E}	28.33±0.04^{c} (45.55±0.06)^{D}	1.71±0.18^{a} (2.74±0.30)^{B}	6.24±0.32^{f} (10.04±0.25)^{F}	20.64±0.33^{c} (31.93±0.51)^{C}	0.697±0.00^{g}	7.47±0.06^{i}
S9	38.23±0.48^{e}	23.53±0.02^{f} (38.09±0.03)^{G}	29.77±0.05^{a} (48.19±0.08)^{B}	1.40±0.10^{b} (2.27±0.16)^{CDE}	7.07±0.21^{e} (11.45±0.17)^{E}	19.50±0.17^{d} (30.17±0.26)^{D}	0.695±0.00^{g}	7.02±0.19^{j}
S10	33.79±0.17^{g}	22.38±0.02^{g} (33.80±0.02)^{J}	28.56±0.01^{b} (43.14±0.02)^{E}	1.36±0.02^{b} (2.06±0.03)^{E}	13.91±0.15^{c} (20.99±0.04)^{C}	17.49±0.26^{f} (27.05±0.41)^{F}	0.705±0.00^{f}	7.59±0.03^{h}
S11	52.54±0.04^{a}	17.18±0.03^{j} (36.20±0.06)^{I}	25.24±0.02^{d} (53.17±0.04)^{A}	1.74±0.05^{a} (3.67±0.11)^{A}	3.3±0.08^{h} (6.05±0.07)^{I}	14.72±0.16^{h} (22.77±0.25)^{H}	0.774±0.00^{a}	7.01±0.02^{j}

*S1 (*Kapi* Satun); S2 (*Kapi* Ranong1); S3 (*Kapi* Ranong2); S4 (*Kapi* Krabi1); S5 (*Kapi* Krabi2); S6 (*Kapi* Songkhla1); S7 (*Kapi* Songkhla2); S8 (*Kapi* Samut Sakorn); S9 (*Kapi* Rayong); S10 (*Kapi* Chachoengsao); S11 (*Kapi* Samut Songkram).

Values in parentheses indicate the content expressed, based on dry weight basis.

Values are given as mean ± SD (n=3). Different superscripts in the same column indicate the significant difference ($p < 0.05$).

Water activity (A_w) of *Kapi* was in the range of 0.669-0.774, with corresponding low moisture content (Table 7). A_w can be related to the consistency of *Kapi*, which varied from soft and pasty to dry and hard. A_w of the final product would depend on how long the sample was allowed to dry under the sun. Furthermore, high salt level directly lowered A_w of products. *Kapi* can be classified as an intermediate moisture food, with an A_w of about 0.7 (Fennema, 1996). Lowered A_w could be associated with the prolonged shelf-life of *Kapi* (Goulas and Kontominas, 2005). The samples had a pH range of 7.01–8.4. The slightly basic pH might have been caused by the degradation products generated during fermentation or the formation of volatile base compounds such as ammonia. When the hydrolysis proceeded, the small proteins or peptides might undergo decomposition, e.g., via deamination with ease. This could favor the formation of low-molecular weight basic compounds.

2.5.2 Formal nitrogen, ammonia nitrogen and amino nitrogen contents

Formal nitrogen contents of different *Kapi* are shown in Table 8. Formal nitrogen content varied from 11.96 to 22.87 mg N/g sample. Generally, the formal nitrogen content has been used to indicate the degree of protein hydrolysis (Chaveesak *et al.,* 1993). Formaldehyde can react with alpha amino group and ammonia, releasing the proton which can be titrated with alkaline solution. Thus, formal nitrogen content can be the indicator for the level of the cleavage of peptides (Angeles and Garcia-Carreno, 2002). The highest formal nitrogen contents were found in S6 (*Kapi* Songkhla1) and S8 (*Kapi* Samut Sakorn) ($p < 0.05$). The lowest value was found in S10 (*Kapi* Chachoengsao) and S11 (*Kapi* Samut Songkram) ($p < 0.05$).

Ammonia nitrogen contents of all samples were in the range of 0.56-0.99 mg N/g sample (Table 8). The ammonia nitrogen content indicates the breakdown of protein and peptides into free amino acid and volatile nitrogen (Lopetcharat *et al.,* 2001; Binsan *et al.,* 2008). Therefore, the differences in ammonia nitrogen contents among samples might be related with different proteolytic activity, both endogenous proteinase and microbial proteinase, during fermentation. The volatile nitrogenous compounds might contribute to different characteristics of the products, especially flavor. S11 (*Kapi* Samut Songkram) had the highest ammonia nitrogen content ($p < 0.05$).

Table 8. Formal nitrogen, ammonia nitrogen, amino nitrogen contents and degree of hydrolysis of various commercial *Kapi*

Samples*	Formal nitrogen content (mg-N/g sample)	Ammonia nitrogen content (mg-N/g sample)	Amino nitrogen content (mg-N/g sample)	Degree of hydrolysis (%)
S1	15.42 ± 0.35^{f}	0.56 ± 0.01^{f}	14.85 ± 0.35^{f}	16.61 ± 0.03^{d}
S2	17.62 ± 0.15^{e}	0.67 ± 0.03^{e}	16.95 ± 0.24^{e}	19.18 ± 0.60^{c}
S3	21.03 ± 0.97^{c}	0.59 ± 0.01^{f}	20.44 ± 0.97^{c}	20.08 ± 0.29^{b}
S4	21.89 ± 0.22^{b}	0.71 ± 0.02^{de}	21.18 ± 0.20^{b}	20.22 ± 0.04^{b}
S5	19.43 ± 0.58^{d}	0.60 ± 0.03^{f}	18.83 ± 0.58^{d}	19.29 ± 0.15^{c}
S6	22.87 ± 0.27^{a}	0.88 ± 0.02^{b}	21.99 ± 0.26^{a}	20.76 ± 0.15^{a}
S7	21.40 ± 0.33^{bc}	0.81 ± 0.06^{c}	20.59 ± 0.34^{bc}	20.1 ± 0.08^{b}
S8	22.78 ± 0.28^{a}	0.69 ± 0.02^{de}	22.08 ± 0.24^{a}	20.76 ± 0.21^{a}
S9	14.73 ± 0.17^{f}	0.81 ± 0.01^{c}	13.93 ± 0.17^{g}	16.49 ± 0.09^{d}
S10	12.09 ± 0.01^{g}	0.74 ± 0.02^{d}	11.35 ± 0.01^{h}	12.68 ± 0.24^{e}
S11	11.96 ± 0.31^{g}	0.99 ± 0.01^{a}	10.97 ± 0.33^{h}	12.81 ± 0.16^{e}

*Samples: see Table 7 caption.
Values are given as mean ± SD (n=3). Different lowercase superscripts in the same column indicate the significant difference ($p < 0.05$).

Amino nitrogen contents of *Kapi* from different provinces of Thailand are shown in Table 8. Amino nitrogen content was calculated based on the differences between formal nitrogen content and ammonia nitrogen content. The amino nitrogen content represents the amount of primary amino group of the sample. An increase in amino nitrogen content is related to the degradation of polypeptide (Lopetcharat *et al.,* 2001; Binsan *et al.,* 2008). Generally, amino nitrogen content was in agreement with the formal nitrogen content. The result reconfirmed that higher cleavage of peptides was obtained in S6 (*Kapi* Songkhla1) and S8 (*Kapi* Samut Sakorn).

2.5.3 Degree of hydrolysis

Degree of hydrolysis (DH) of all samples varied (Table 8). DH is the measure of the extent of cleavage of peptide linkages. DH close to 100 % means that all proteins in the sample are completely hydrolyzed to free amino acids (Panyam and

Kilara, 1996). DH of the samples was from 12.68 to 20.76%. DH was in agreement with formal nitrogen content and amino nitrogen content. S6 (*Kapi* Songkhla1) and S8 (*Kapi* Samut Sakorn) showed the highest DH (20.76%) and S10 (*Kapi* Chachoengsao) and S11 (*Kapi* Samut Songkram) had the lowest DH (12.68 and 12.81%, respectively). Due to different raw materials, processing condition and time of fermentation as well as different types or levels of proteolytic enzymes, varying degrees of cleavage were presumed. Products with different DH might possess different characteristics, taste, flavor, color and other attributes. Free amino groups available were able to undergo several reactions, e.g., Maillard reaction, thereby affecting the color or browning of products.

2.5.4 Protein patterns

Protein patterns of different *Kapi* samples are shown in Figure 1. All samples had proteins or peptides with molecular weight lower than 100 kDa. Major proteins were found in the range of 10-15 kDa. It was noted that *Kapi* Samut Songkram (S11) had the proteins with MW of 65 and 30 kDa. This coincided with the lowest DH of those samples. The results indicated the differences in protein pattern in individual sample. For all samples, there were no myosin heavy chain (MHC) and actin bands retained. This suggested that MHC and actin underwent degradation completely through proteolysis during fermentation in all samples. It has been reported that MHC was the most susceptible muscle proteins (Benjakul *et al.,* 1997). When MHC was not available, actin can be hydrolyzed (Riebroy *et al.,* 2008). It was also found that troponin and tropomyosin also disappeared in all samples. Krill and shrimp were found to contain serine protease and metalloprotease (Garcia-Carreno *et al.,* 1994). Those proteases along with proteases from halophilic bacteria more likely played a role in hydrolysis of proteins in raw material. With intensive degradation, free amino acids or low-molecular weight peptides can be produced. The formation of low-molecular weight peptides or proteins was in accordance with the increasing DH, formal nitrogen and amino nitrogen contents (Table 8).

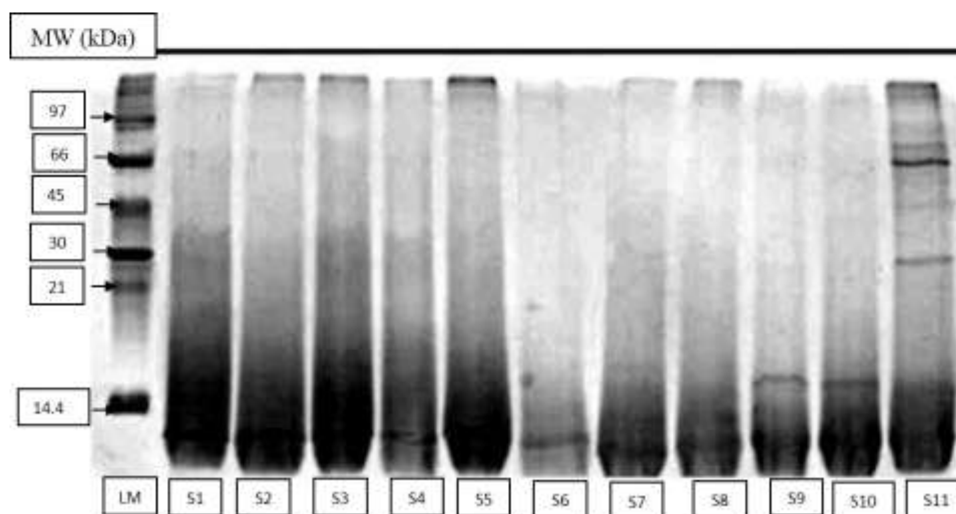

Figure 1. Protein patterns of various commercial *Kapi.* LM low-molecular weight marker. Samples: see Table 7 caption.

2.5.5 Carotenoid

2.5.5.1 Carotenoid content

Total carotenoid contents in different *Kapi* are shown in Table 9. Carotenoids, especially astaxanthin, provide the desirable reddish-orange color in crustaceans (Higuera-Ciapara *et al.,* 2006). Carotenoid content of *Kapi* varied from 0.54 to 1.97 mg/g lipids. Among all samples, S7 (*Kapi* Songkhla2) had the highest total carotenoid content (1.97 mg/g lipids) ($p < 0.05$), whereas S11 (*Kapi* Samut Songkram) (0.54 mg/g lipids) and S6 (*Kapi* Songkhla1) (0.69 mg/g lipids had the lowest carotenoid content ($p < 0.05$). The differences in carotenoid content could plausibly be to the different raw materials used. Shrimp with different species and feed used showed varying carotenoid content (Shahidi and Brown, 1998). Supplementation of carotenoids in red porgy (*Pagrus pagrus*) could increase redness, yellowness and chroma of their skin (Manganaro *et al.,* 2012). Additionally, stability of carotenoid in different samples could be varied, depending on the process or other factors involved. Carotenoid content of hepatopancreas of Pacific white shrimp was 336.40 mg/kg sample (Takeungwongtrakul *et al.,* 2012a). Sachindra *et al.* (2006) reported the highest carotenoid content (43.9 mg/kg sample) from head and carapace of *Penaeus indicus*

when 50:50 (v/v) mixture of hexane and isopropanol was used as extracting medium. The variation in carotenoids in crustaceans is caused by various factors such as species, feed, parts of body, environmental condition and other variables (Mezzomo *et al.,* 2011; Takeungwongtrakul *et al.,* 2012b). Apart from protein, carotenoids have paid increasing interest as the important source of antioxidants from crustaceans (De Holanda and Netto, 2006). The presence of carotenoids in *Kapi* indicated that carotenoids were stable during fermentation under the high salt environment. In general, carotenoids were associated with protein as carotenoprotein (Armenta-Lopez *et al.,* 2002). When proteins were degraded, free carotenoids were released and might undergo oxidation to some extent. These carotenoids more likely contributed to color of salted shrimp paste to some degree. Carotenoid in shrimp was reported to possess antioxidative activity (Bustos *et al.,* 2003; Sowmya and Sachindra, 2012).

2.5.5.2 Thin-layer chromatography (TLC)

Carotenoids in *Kapi* were separated by TLC (Figure 2). Similar TLC chromatograms were observed among different samples. The major bands with R_f of 0.33, 0.40 and 0.75 were observed, corresponding to free astaxanthin, canthaxanthin and astaxanthin diester, respectively. The R_f of cantaxanthin was in agreement with that reported by Todd (1998), in which R_f of 0.40 was obtained. Kobayashi and Sakamoto (1999) reported that astaxanthin diester had R_f in range of 0.75-0.85. Shahidi and Brown (1998) and Sachindra *et al.* (2001) also reported that astaxanthin and its esters are the major carotenoids in the marine crustaceans. It was noted that carotenoid in different samples varied in terms of components. No canthaxanthin was found in S11 (*Kapi* Samut Songkram). Also, proportion of those components varied with samples. The results suggested that raw materials used for production of salted shrimp paste were different. As a result, carotenoids in the resulting products varied in composition. This might affect physical properties and bioactivity of products.

Table 9. Carotenoid content and colors of various commercial *Kapi*

Samples*	Carotenoid content**	L^*	a^*	b^*	ΔE^*	ΔC^*
S1	1.41±0.04^{c}	42.37±0.05^{e}	15.39±0.07^{a}	13.49±0.17^{b}	55.27±0.07^{d}	19.55±0.11^{b}
S2	1.19±0.03^{e}	46.74±0.04^{b}	12.13±0.17^{c}	11.86±0.21^{d}	49.92±0.03^{h}	16.05±0.04^{d}
S3	1.32±0.08^{d}	51.84±0.04^{a}	12.68±0.03^{b}	17.59±0.07^{a}	47.10±0.01^{i}	20.76±0.06^{a}
S4	1.35±0.06^{d}	38.5±0.07^{i}	11.3±0.02^{d}	10.87±0.10^{e}	57.35±0.06^{b}	14.76±0.04^{e}
S5	1.95±0.03^{a}	43.37±0.05^{d}	9.7±0.17^{e}	12.03±0.19^{d}	52.58±0.03^{f}	14.54±0.08^{e}
S6	0.69±0.02^{f}	39.34±0.08^{h}	5.24±0.06^{h}	9.91±0.24^{f}	55.38±0.11^{c}	10.29±0.20^{g}
S7	1.97±0.02^{a}	44.65±0.06^{c}	11.81±0.07^{c}	12.92±0.17^{c}	52.05±0.03^{g}	16.59±0.17^{c}
S8	1.53±0.03^{b}	34.13±0.06^{j}	8.34±0.07^{f}	6.39±0.20^{i}	60.43±0.08^{a}	9.59±0.10^{h}
S9	1.51±0.01^{b}	39.91±0.05^{f}	9.75±0.58^{e}	7.6±0.14^{g}	55.16±0.08^{e}	11.45±0.40^{f}
S10	1.43±0.02^{c}	39.36±0.08^{h}	7.46±0.04^{g}	7.21±0.23^{h}	55.24±0.08^{de}	9.46±0.13^{h}
S11	0.54±0.01^{g}	39.53±0.03^{g}	9.43±0.11^{e}	4.56±0.09^{j}	55.15±0.02^{e}	9.56±0.07^{h}

*Samples: see Table 7 caption.

**Carotenoid content was expressed as mg/g lipids.

Values are given as mean ± SD (n=3). Different lowercase superscripts in the same column indicate the significant difference ($p < 0.05$).

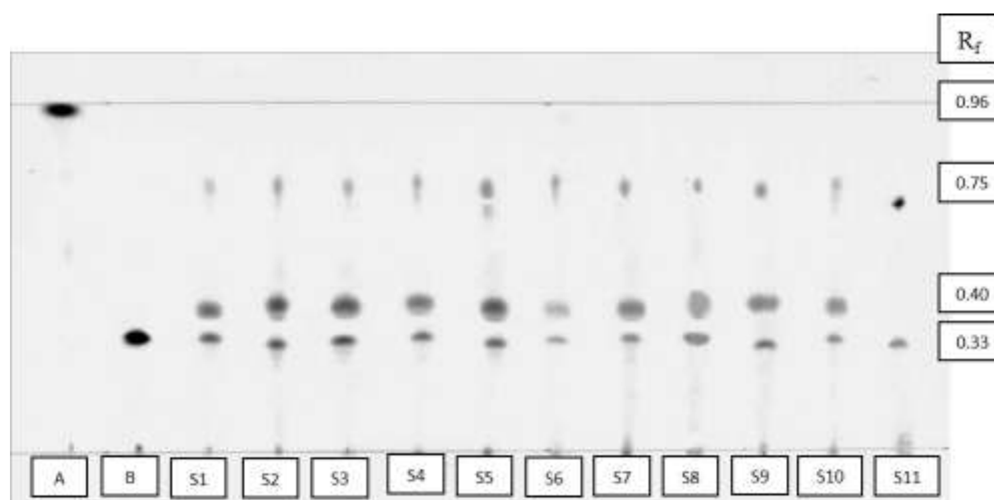

Figure 2. Thin-layer chromatogram of carotenoids from various commercial *Kapi*. (A: β-carotene, B: astaxanthin, S1-S11: *Kapi* samples). Samples: see Table 7 caption.

2.5.6 Color

The different *Kapi* samples had different colors. L^* (lightness), a^* (redness), b^* (yellowness), ΔE^* (total difference in color) and ΔC^* (difference in chroma) were in the range of 34.13-51.84, 5.24-15.39, 4.56-17.59, 47.10-60.43 and 9.46-20.76, respectively. S8 (*Kapi* Samut Sakorn) had the lowest L^* and the highest ΔE^*. The lowest ΔC^* was also noticeable in this sample. This sample was brown in color. On the other hand, S1 (*Kapi* Satun) had the highest a^* value, whereas S3 (*Kapi* Ranong2) showed the highest b^* value ($p < 0.05$). S3 (*Kapi* Ranong2) also exhibited the highest ΔC^* value ($p < 0.05$). The difference in color might be caused by the different pigment contents, as determined by raw materials, process as well as ingredients added. Highest a^* value for *Kapi* Satun was in agreement with the highest total carotenoid content (Table 9). Generally, lipid oxidation was associated with browning mediated by Maillard reaction (Yarnpakdee *et al.,* 2012). The carbonyl groups of aldehydes and ketone, the oxidation products, could react with amino groups of free amino acids or peptides generated during hydrolysis, leading to yellow or brown color development (Yarnpakdee *et al.,* 2014). DH in each product might, therefore, be related with different colors.

2.6 Conclusion

Different *Kapi* had varying compositions. However, protein constituted the major component in all samples. Hydrolysis of proteins took place at different degrees, leading to varying protein or peptide. Carotenoids were also found as the components, which partially contributed to color. Studies on sensory properties, and flavor profile of *Kapi* will be further investigation, in which desirable characteristic for this product could be gained.

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CHAPTER 3

CHEMICAL COMPOSITION, SENSORY AND ANTIOXIDATIVE PROPERTIES OF SALTED SHRIMP PASTE (*KAPI*) IN THAILAND

3.1 Abstract

Chemical compositions, sensory and antioxidative properties of 11 salted shrimp paste (*Kapi*) obtained from various places of Thailand were determined. Different *Kapi* samples had varying amino acid compositions. Glu/Gln and Asp/Asn were the major amino acids. Among all samples, S9 (*Kapi* Rayong), which had the highest total amino acid (68.95 mg/g sample), generally had the highest sensory score for all attributes. Volatile compounds varied in types and abundance among samples, but pyrazine derivatives were the major volatile components in all samples. Browning intensity and intermediate browning products were different between samples. The highest antioxidative activities as determined by DPPH, ABTS, H_2O_2 radical and singlet oxygen scavenging activities, FRAP and metal chelating activity were found for S1 (*Kapi* Satun). Therefore, salted shrimp pastes having nutritive value and antioxidative activity were different in sensory property, thereby determining the consumer acceptability.

3.2 Introduction

Kapi is a traditional salted shrimp paste of Thailand. It is mainly produced from the marine shrimp or krill (*Acetes* or *Mesopodopsis species*), which are mixed with salt at a ratio of 3-5:1. The moisture content is decreased by sun drying, and then it is thoroughly blended or homogenized to produce semi-solid paste. The paste is fermented for one months until the desired flavor is developed (Phithakpol, 1993). *Kapi* is usually used as a condiment to enhance the palatability of foods (Yoshida, 1998). *Kapi* is very rich in umami taste and contains high amounts of free glutamic acid (647 mg/100 g) (Mizutani *et al.,* 1987). This product has slight cheese-like flavor and an appetite-stimulating aroma (Peralta *et al.,* 2008). More than 150 volatile compounds

have been identified in fish and shrimp pastes (Cha *et al.,* 1998). The compounds consist of aldehydes, ketones, alcohols, aromatic compounds, *N*-containing compounds, esters, *S*-containing compounds and some other compounds. Previous studies noted that the presence of these *S*-containing compounds may affect the overall flavor because of their low thresholds (Maga and Katz, 1979; Agrahar-Murugkar and Subbulakshmi, 2006). During fermentation, the transformation of organic substances into simpler compounds such as peptides, amino acids, and other nitrogenous compounds either by the action of microorganisms or endogenous enzymes takes place. Peptides and amino acids are important contributors to the flavor and aroma of fermented products (Raksakulthai and Haard, 1992). Furthermore, the fermented fish products containing active peptides or free amino acids generated throughout fermentation from both endogenous and exogenous enzymes (Rajapakse *et al.,* 2005). Recently, some fermented shrimp and krill products have been reported to exhibit strong antioxidant activities (Faithong *et al.,* 2010). However, a little information regarding amino acid compositions, volatile compounds, antioxidative activities and sensory properties of *Kapi* (salted shrimp paste) produced in Thailand has been reported. Thus, the objective of this study was to determine chemical composition, sensory and antioxidative properties of *Kapi* collected from various regions of Thailand.

3.3 Objective

To determine chemical composition, sensory and antioxidative properties of *Kapi* collected from various regions of Thailand.

3.4 Materials and methods

3.4.1 Chemicals

All chemicals were of analytical grade. 2,4,6-trinitrobenzene-sulphonic acid (TNBS), 2,20-azinobis-(3-thylbenzothiazoline-6-sulphonic acid) (ABTS), 1,1-diphenyl-2-picrylhydrazyl (DPPH), 2,4,6 tripyridyltriazine (TPTZ), 3-(2-pyridyl)- 5,6-diphenyl-1,2,4-triazine-4’,4’’-disulphonic acid sodium salt (ferrozine), hydrogen peroxide (H_2O_2), ethylenediaminetetraacetic acid (EDTA), 5,5-dimethyl-1-pyrroline

N-oxide (DMPO), N,N-dimethyl ρ-nitrosoaniline (DPN), sodium hypochlorite (NaOCl), histidine and 6-hydroxy 2,5,7,8-tetram-ethylchroman-2-carboxylic acid (Trolox) were purchased from Sigma Chemical Co. (St. Louis, MO, USA).

3.4.2 Collection and preparation of samples

Kapi samples were purchased from different provinces in Thailand, including Sa-tun (1 sample) [S1], Ranong (2 samples) [S2, S3], Krabi (2 samples) [S4, S5], Songkhla (2 samples) [S6, S7], Samut Sakhon (1 sample) [S8], Rayong (1 sample) [S9], Chachoengsao (1 sample) [S10] and Samut Songkram (1 sample) [S11]. Those *Kapi* were produced by using different type of raw material including krill (*Mesopodopsis* spp.): S4, S5, S8, S9 and S11, shrimp (*Acetes* spp.): S1, S2, S3, S6 and S7 as well as and shrimp (*Mysis* spp.): S10. sample was separated into several portions (100 g each), placed in polyethylene bag and heat-sealed. The samples were kept at -20°C and the storage time was not longer than 2 months. All samples were subjected to analyses.

3.4.3 Determination of amino acid compositions

Amino acid compositions of *Kapi* were determined according to the method of Minh Tauy *et al.* (2014) with a slight modification. Twenty milligrams of sample were hydrolyzed in 6 M HCl at 110°C for 22 h under vacuum. The hydrolysate was neutralized with 6 M and 0.6 M NaOH, and filtered through a cellulose membrane filter (0.45 μm; Toyo Roshi Kaisha, Ltd., Tokyo, Japan). The filtrate was used for amino acid analysis using an amino acid analysis system (Prominence; Shimadzu, Kyoto, Japan) equipped with a column (Shim-pack Amino- Li, 100 mm×6.0 mm i.d.; column temperature, 39.0°C; Shimadzu) and pre-column (Shim-pack ISC-30/S0504 Li, 150 mm×4.0 mm i.d.; Shimadzu). Amino acids were detected using a fluorescence detector (RF-10AXL; Shimadzu, Kyoto, Japan).

3.4.4 Determination of volatile compounds

The volatile compounds of different *Kapi* samples were determined using a solid-phase microextraction gas chromatography mass spectrometry (SPME

GC-MS) following the method of Iglesias and Medina (2008) with a slight modification.

3.4.4.1 Extraction of volatile compounds by SPME fiber

To extract volatile compounds, 5 g of *Kapi* was mixed with 10 ml of deionized water. The mixture was homogenized at a speed of 13,000×*g* for 1 min to disperse the sample. The homogenate was placed in a 20-ml headspace vial (Supelco, Bellefonte, PA, USA) for each SPME. The vials were tightly capped with a PTFE septum and heated at 60°C with equilibrium time of 10 h. The SPME fiber (50/30 lm DVB/Carboxen™/PDMS StableFlex™ (Supelco, Bellefonte, PA, USA) was conditioned at 270°C for 15 min before use and then exposed to the headspace. The 20 ml-vials (Agilent Technologies, Palo Alto, CA, USA) containing the sample extract and the volatile compounds were allowed to absorb into the SPME fiber at 60°C for 1 h. The volatile compounds were then desorbed in the GC injector port for 15 min at 270°C.

3.4.4.2 GC-MS analysis

GC-MS analysis was performed in a HP 5890 series II gas chromatography (GC) coupled with HP 5972 mass-selective detector equipped with a splitless injector and coupled with a quadrupole mass detector (Hewlett Packard, Atlanta, GA, USA). Compounds were separated on a HP-Innowax capillary column (Hewlett Packard, Atlanta, GA, USA) (30 m±0.25 mm ID, with film thickness of 0.25 μm). The GC oven temperature program was: 35ºC for 3 min, followed by an increase of 3°C/min to 70ºC, then an increase of 10ºC/min to 200ºC, and finally an increase of 15ºC/ min to a final temperature of 250ºC and holding for 10 min. Helium was employed as a carrier gas, with a constant flow of 1 ml/min. The injector was operated in the splitless mode and its temperature was set at 270ºC. Transfer line temperature was maintained at 260ºC. The quadrupole mass spectrometer was operated in the electron ionization (EI) mode and source temperature was set at 250ºC. Initially, full scan-mode data was acquired to determine appropriate masses for the later acquisition in scan mode under the following conditions: mass range: 25-500 amu and scan rate:

0.220 s/scan. All analyses were performed with ionization energy of 70 eV, filament emission current at 150 μA, and the electron multiplier voltage at 500 V.

3.4.4.3 Analyses of volatile compounds

Identification of the compounds was done by consulting ChemStation Library Search (Wiley 275.L). Quantitative determination was carried out using an internal calibration curve that was built using stock solutions of the compounds in ultra-pure water saturated in salt and analyzing them by the optimized HS-SPME method. Quantification limits were calculated to a signal-to-noise (S/N) ratio of 10. Repeatability was evaluated by analyzing 3 replicates of each sample. The identified volatile compounds were presented in the term of abundance.

3.4.5 Sensory properties

Samples were evaluated by 50 untrained panelists, who consume *Kapi* regularly. The samples were wrapped with aluminum foil and heated in hot air oven at 60°C for 30 min. Samples were cut to obtain a thickness of 1 cm (2×2 cm^2), then served in white paper plate at room temperature. All samples was coded with three digit random numbers and divided into 3 groups (4, 4 and 3 samples). Each group was randomly served. The panelists were allowed to rest for at least 15 min between different groups. Panelists were instructed to rinse their mouths with water, cucumber and cracker between different samples. Evaluations were made in individual sensory evaluation booths under fluorescent white light. The panelists were asked to assess samples for appearance liking, color liking, odor liking, flavor liking, texture liking and overall liking using a 9-point hedonic scale (1 = dislike extremely, 9 = like extremely) (Mellgard *et al.,* 2007).

3.4.6 Browning and Maillard reaction product

3.4.6.1 Preparation of water extract

The extract was prepared according to the method of Peralta *et al.* (2008) with a slight modification. *Kapi* (2 g) was mixed with 50 ml of distilled water. The mixtures were homogenized using an IKA Labortechnik homogenizer (Selangor,

Malaysia) at a speed of 10,000×*g* for 2 min. The homogenates were then subjected to centrifugation at 13,000×*g* for 15 min at room temperature (Model RC-B Plus centrifuge Newtown, CT, USA). The supernatant was collected. The pellet was re-extracted as described above. The supernatants were combined and adjusted to 50 ml using distilled water.

3.4.6.2 Measurement of absorbance at 280 and 295 nm

A_{280} and A_{295} of the extract were determined according to the method of Ajandouz *et al.* (2001). The absorbance of the appropriately diluted extract was measured at 280 and 295 nm using UV-1601 spectrophotometer (Shimadzu, Kyoto, Japan) to monitor the formation of Maillard reaction intermediate products.

3.4.6.3 Measurement of browning intensity

The browning intensity of the extract was measured according to the method of Benjakul *et al.* (2005). Appropriate dilution was made using distilled water and the absorbance was measured at 420 nm using UV-1601 spectrophotometer.

3.4.6.4 Measurement of fluoresence intensity

Fluorescent intermediate products from Maillard reaction in the extract were determined as described by Morales and Jimenez-Perez (2001). The fluorescence intensity of appropriately diluted extract was measured at an excitation wavelength of 347 nm and emission wavelength of 415 nm using a fluorescence spectrophotometer RF-1501 (Shimadzu, Kyoto, Japan).

3.4.7 Antioxidative properties

Water extract from different *Kapi* were subjected to determination of antioxidative activity using various assays.

3.4.7.1 DPPH radical scavenging activity

DPPH radical scavenging activity was determined according to the method of Wu *et al.* (2003) with a slight modification. The extract (1.5 ml) was added

with 1.5 ml of 0.15 mM 2,2-diphenyl- 1 picrylhydrazyl (DPPH) in 95% ethanol. The mixture was then mixed vigorously and allowed to stand for 30 min in dark at room temperature. The resulting solution was measured at 517 nm using an UV-1601 spectrophotometer. The blank was prepared in the same manner except that distilled water was used instead of the sample. The standard curve was prepared using Trolox in the range of 10-60 μM. The activity was expressed as μmol Trolox equivalents (TE)/g sample.

3.4.7.2 ABTS radical scavenging activity

ABTS radical scavenging activity was determined as described by Amao *et al.* (2001) with a slight modification. The stock solutions included 7.4 mM ABTS solution and 2.6 mM potassium persulfate solution. The working solution was prepared by mixing two stock solutions in equal quantities and allowed them to react in the dark for 12 h at room temperature. The solution was then diluted by mixing 1 ml of ABTS solution with 50 ml of methanol to obtain an absorbance of 1.1 (±0.02) at 734 nm using an UV-1601 spectrophotometer. ABTS solution was prepared freshly for each assay. To initiate the reaction, 150 μl of sample was mixed with 2.85 ml of $ABTS^{\bullet+}$ solution. The mixture was incubated at room temperature for 2 h in dark. The absorbance was then read at 734 nm using an UV-1601 spectrophotometer. A Trolox standard curve (50-600 μM) was prepared. Distilled water was used instead of the sample and prepared in the same manner to obtain the control. ABTS radical scavenging activity was expressed as μmol Trolox equivalents (TE)/g sample.

3.4.7.3 Ferric reducing antioxidant power (FRAP)

FRAP was evaluated by the method of Benzie and Strain (1996). The stock solutions included 10 mM TPTZ (2,4,6-tripyridyl-s-triazine) solution in 40 mM HCl, 20 mM $FeCl_3.6H_2O$ solution and 300 mM acetate buffer (pH 3.6). The working solution was prepared freshly by mixing 25 ml of acetate buffer, 2.5 ml of TPTZ solution and 2.5 ml of $FeCl_3.6H_2O$ solution. The mixture was incubated at 37°C for 30 min and was referred to as FRAP solution. The sample (150 μl) was mixed with 2.85 ml of FRAP solution. The mixture was allowed to stand in dark for 30 min at room

temperature. Ferrous tripyridyltraizine complex, colored product, was measured by reading the absorbance at 593 nm. The standard curve was prepared using Trolox ranging from 50 to 600 μM. The activity was expressed as μmol Trolox equivalents (TE)/g sample.

3.4.7.4 Metal chelating activity

Metal chelating activity was investigated as described by Decker and Welch (1990) with a slight modification. Sample (220 μl) was mixed with 5 μl of 2 mM $FeCl_2$ and 10 μl of 5 mM ferrozine. The mixture was allowed to stand at room temperature for 20 min. Absorbance at 562 nm was read. EDTA with the concentrations of 0-30 μM was used as standard. Metal chelating activity was expressed as μmol EDTA equivalent (EE)/g sample.

3.4.7.5 Hydrogen peroxide radical scavenging activity

Hydrogen peroxide scavenging activity was assayed according to the method of Kittiphattanabawon *et al.* (2012). The extract (3.4 ml) was mixed with 600 μl of 43 mM hydrogen peroxide in 0.1 M phosphate buffer (pH 7.4). The absorbance at 230 nm of the reaction mixture was recorded after 40 min of reaction at 25°C. For sample blank, hydrogen peroxide was omitted and replaced by 0.1 M phosphate buffer (pH 7.4). Trolox (0-10 mM) was used as standard. The hydrogen peroxide scavenging activity was expressed as μmol Trolox equivalents (TE)/g sample.

3.4.7.6 Singlet oxygen scavenging activity

Singlet oxygen scavenging activity was determined as described by Kittiphattanabawon *et al.* (2012). The chemical solutions and the extract were prepared in 45 mM sodium phosphate buffer (pH 7.4). The reaction mixture consisted of 0.4 ml of extract, 0.5 ml of 200 μM *N,N*-dimethyl para-nitro soaniline (DPN), 0.2 ml of 100 mM histidine, 0.2 ml of 100 mM sodium hypochlorite, and 0.2 ml of 100 mM H_2O_2. Thereafter, the total volume was made up to 2 ml with 45 mM sodium phosphate buffer (pH 7.4). The absorbance of the reaction mixture was measured at 440 nm after incubation at room temperature (25°C) for 40 min. Sample blank was run for each

sample in the same manner, except DPN, histidine, and NaOCl solutions were replaced by sodium phosphate buffer. A standard curve of Trolox (0-10 mM) was prepared. Singlet oxygen scavenging activity was expressed as μmol Trolox equivalents (TE)/g sample.

3.4.8 Statistical analysis

All analyses were conducted in triplicate. Statistical analysis was performed using one-way analysis of variance (ANOVA). Mean comparison was carried out using Duncan's multiple range test (Steel *et al.,* 1980). SPSS statistic program (Version 10.0) (SPSS, 1.2, 1998) was used for data analysis.

3.5 Results and discussion

3.5.1 Amino acid compositions

Amino acid compositions of 11 *Kapi* samples are presented in Table 10. Total amino acid content varied among the samples. S9 (*Kapi* Rayong) had the highest total amino acid (68.95 mg/g sample). Coincidentally, the highest total essential amino acid content (25.16 mg/g sample) was also found for S9. In general, Glu/Gln and Asp/Asn were the major amino acids in salted shrimp paste. Gly, Leu and Lys were also found at a high extent in all samples. Xu *et al.* (2008) reported that fish sauce produced from squid by-product was rich in Glu, Asp, Cys, Leu and Ala (12.10, 9.33, 8.44, 7.32 and 7.22 mg/g sample respectively). The differences in amino acid compositions among the samples were more likely due to the difference in fermentation and processes used. Differences in raw material, especially shrimp or krill, were also presumed. Amino acids mainly contributed significantly to the taste and odor of salted shrimp paste. The typical flavor of Glu is meaty (Xu *et al.,* 2008). Taste of salted shrimp paste was influenced by Glu for umami and by Asp for sweetness (Kim *et al.,* 2005). Gly, Ala, Ser and Thr are also associated with sweetness (Liu, 1989). The contribution of amino acids to the aroma of fish sauce was reported by Lopetcharat *et al.* (2001). Based on the result, *Kapi* could be an excellent source of amino acids, particularly essential amino acids. Additionally, those amino acids more likely contributed to taste and flavor of *Kapi*.

Table 10. Amino acid composition of various commercial *Kapi*

Amino acid composition (mg/g sample)	S1*	S2	S3	S4	S5	S6	S7	S8	S9	S10	S11
Alanine (Ala)	2.57	1.93	4.59	1.69	4.03	5.01	3.26	3.74	6.65	4.66	4.36
Arginine (Arg)	1.69	0.77	2.55	2.13	3.70	1.16	0.66	2.21	2.49	0.91	0.77
Aspartic acid and Asparagine (Asp/Asn)	2.99	2.29	7.31	2.64	5.42	6.32	3.74	4.57	7.10	4.36	5.21
Cysteine (Cys)	0.06	0.08	0.17	0.05	0.13	0.18	0.11	0.11	0.04	0.14	0.12
Glycine (Gly)	1.87	1.52	3.86	1.33	2.79	4.29	2.33	2.79	4.48	2.76	3.06
Glutamic acid and Glutamine (Glu/Gln)	0.25	4.12	15.43	7.87	16.39	9.39	6.01	6.27	12.08	10.17	11.40
Histidine (His)[B]	0.46	0.37	0.83	0.36	0.71	0.54	0.51	0.59	0.98	0.74	0.64
Hydrolysine (Hyl)	0.01	0.01	0.02	0.01	0.02	0.05	0.05	0.03	0.06	0.03	0.03
Hydroxyproline (Hyp)	0	0	0	0	0	0	0	0	0	0	0
Isoleucine (Ile)[A]	1.45	1.27	3.07	1.23	2.78	3.19	1.74	2.13	4.11	2.43	2.63
Leucine (Leu)[A]	2.58	2.08	4.10	1.79	4.67	5.14	3.08	3.39	6.42	3.62	3.99
Lysine (Lys)[A]	2.23	1.99	3.76	1.69	3.97	4.58	2.73	3.11	6.23	3.26	3.49
Methionine (Met)[A]	0.55	0.74	1.47	0.57	1.46	1.69	1.05	1.22	1.73	1.31	1.24
Phenylalanine (Phe)[A]	1.49	1.23	2.48	1.25	2.46	2.76	1.54	1.74	2.61	1.67	2.26
Proline (Pro)	3.45	2.66	5.80	1.90	4.21	4.58	3.74	3.4	5.68	3.87	3.30
Serine (Ser)	0.60	0.40	0.83	0.42	1.84	0.61	0.20	1.23	1.62	1.21	0.71
Tryptophan (Trp)[A]	0	0.03	0.02	0	0.05	0	0	0	0	0	0
Tyrosine (Tyr)	1.19	1.03	2.00	0.71	2.00	2.46	1.54	1.74	2.61	1.67	2.26
Valine (Val)[A]	1.41	1.25	2.98	1.12	2.66	3.04	1.65	2.14	4.06	2.53	2.33
Total EAA[C]	9.71	8.59	17.88	7.65	18.05	20.40	11.79	13.73	25.16	14.82	15.94
Total NEAA[D]	14.68	14.81	42.56	18.75	40.53	34.05	21.64	26.09	42.81	29.78	31.22
Total amino acid	24.85	23.77	61.27	26.76	59.29	54.99	33.94	40.41	68.95	45.34	47.80

*Samples: see Table 7 caption.

A: Essential amino acid in adults. B: Essential amino acid in children. C: Essential amino acid. D: Non-essential amino acid.

3.5.2 Volatile compounds

Volatile compounds of different salted shrimp samples produced in Thailand were determined using a solid-phase microextraction gas chromatography mass spectrometry (SPME GC-MS). Different volatile compounds were identified. Those consisted of alcohols, aldehydes, ketones, hydrocarbon and nitrogen-containing compounds (Table 11).

Nitrogen-containing compounds, especially pyrazine derivatives, seemed to be the major volatile components in salted shrimp pastes. 2,5-dimethyl pyrazine, 2,6-dimethyl pyrazine, 3-ethyl, 2,5 dimethyl pyrazine and 6-ethyl, 2,3,5-trimethyl pyrazine were found in all samples. Pyrazines were reported to contribute to nutty, roasted and toasted aromas in many foods (Wong and Bernhard, 1988). Sanceda *et al.* (1990) found that pyrazines could be responsible for the burnt and sweet odors of Vietnamese fish sauce (*nouc-mam*). Pyrazines were reported to be formed by Maillard reaction through strecker degradations from various nitrogen sources such as amino acids (Jaffres *et al.,* 2011). Slightly high pH of shrimp paste (pH 7.2-8.4) could favor the formation of pyrazine (Sanceda *et al.,* 1990). S6 (*Kapi* Songkhla1) showed the highest abundance in 2,5-dimethyl-pyrazines, whereas S8 (*Kapi* Samut Sakorn) exhibited the highest level of 2,6-dimethyl-pyrazines. S6 (*Kapi* Songkhla1) had the highest abundance in 3-ethyl-2,5-dimethyl-pyrazines and 2,3,5-trimethyl-6-ethyl-pyrazine.

2-butanol and 3-methyl-butanol were found at high abundance in most samples. Michihata *et al.* (2002) noted that butanol derivatives might be formed by microbial fermentation, especially regulated by lactic acid bacteria. 1-hexanol, 1-penten-3-ol, 1-octen-3-ol and benzeneethanol were also found in most samples. Those alcohols might be the degradation products from lipid oxidation. 3-methyl-butanol was dominant in S9 (*Kapi* Rayong) and S10 (*Kapi* Chachoengsao), whereas 2 butanol was highest in S5 (*Kapi* Krabi2). Furthermore, other alcohols varied with samples. 2-ethyl, 1 hexanol was found only in S1 (*Kapi* Satun), while 1-octen-3-ol was dominant in S10 (*Kapi* Chachoengsao).

Table 11. Volatile compounds of various commercial *Kapi*

Volatile compounds	Peak area (Abundance) × 10^5										
	S1*	S2	S3	S4	S5	S6	S7	S8	S9	S10	S11
Nitrogen-containing compounds											
Methyl-pyrazine	709	714	ND	ND	ND	1521	ND	925	ND	805	ND
Ethyl-pyrazine	ND	ND	ND	ND	ND	703	ND	ND	ND	ND	ND
Trimethyl-pyrazine	4833	3100	ND	ND	ND	ND	ND	ND	ND	ND	1895
Tetramethyl-pyrazine	1780	476	ND	436	368	4538	795	ND	ND	672	1596
2,5-dimethyl-pyrazine	3301	3427	3163	2315	5198	22098	3901	6470	2206	4568	954
2,6-dimethyl-pyrazine	1149	2831	2128	3401	7017	15345	3250	25357	2367	3948	1167
2-ethyl-6-methyl-pyrazine	358	537	ND	528	628	4835	ND	1988	408	1168	ND
3-ethyl-2,5-dimethyl-pyrazine	1842	2042	2630	1214	3766	59015	2273	2702	2507	4568	338
2-ethyl-3,5-dimethyl-pyrazine	2418	ND	ND	ND	ND	ND	1573	ND	ND	ND	ND
2,3-diethyl, 5-methyl-pyrazine	ND	ND	ND	ND	ND	7155	ND	ND	ND	1883	ND
2,3,5-Trimethyl-6-ethyl-pyrazine	869	315	2960	1837	3642	17713	2067	2796	1204	3026	342
Alcohols											
Furanmethanol	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	793
2-methyl, 1- propanol	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	470
3-methylthio, 1-propanol	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	262
1-butanol	141	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
2-butanol	2626	11302	11427	9666	51622	6871	10567	5633	982	7701	11917
3-methyl-butanol	19862	10329	9589	ND	7548	14042	ND	ND	20973	20147	14689
1-Pentanol	ND	1485	ND	ND	ND	1820	999	ND	ND	1213	1133

*Samples: see Table 7 caption.
ND: non-detectable.

Table 11. Volatile compounds of various commercial *Kapi* (Cont.)

Volatile compounds	Peak area (Abundance) × 10^5										
	S1*	S2	S3	S4	S5	S6	S7	S8	S9	S10	S11
1-Penten-3-ol	ND	2742	ND	641	1591	1282	2541	1880	2023	2310	1702
1-Pentaethiol	ND	ND	2735	ND	ND	ND	ND	ND	ND	ND	ND
1-Hexanol	ND	1121	881	294	527	2314	1195	ND	ND	701	ND
2-ethyl, 1-hexanol	3922	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
Cyclohexanol	514	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
4-hepten-1-ol	ND	382	ND	ND	ND	ND	ND	ND	ND	ND	ND
cis-hept-4-enol	ND	ND	366	ND	ND	ND	ND	ND	ND	ND	ND
1-Octanol	253	ND	ND	ND	ND	2262	ND	ND	219	ND	ND
2-Octen-1-ol	ND	235	ND	ND	ND	ND	ND	ND	ND	ND	ND
2,7- Octadiene-1-ol	142	393	ND	ND	ND	312	ND	ND	ND	ND	ND
1-Octen-3-ol	ND	2682	835	606	1553	ND	1950	ND	ND	3963	ND
3-methyl-phenol	ND	ND	ND	ND	ND	388	ND	ND	ND	ND	ND
Aldehydes											
Pentanal	ND	ND	ND	ND	ND	788	ND	ND	1594	1790	ND
Hexanal	ND	698	36	33829	43	ND	ND	ND	ND	ND	ND
4-heptenal	ND	1102	ND	ND	ND	ND	ND	ND	ND	ND	ND
2-Octenal	ND	3789	ND	ND	ND	ND	ND	ND	1939	ND	ND
Benzaldehyde	4900	27352	18088	19763	12030	23931	5096	8234	5177	6375	1143
Ketones											
1-phenyl-ethanone	ND	503	ND	ND	ND	ND	ND	ND	ND	ND	ND
1,2-diphenyl-ethanone	ND	ND	ND	ND	ND	2091	ND	ND	ND	ND	ND

*Samples: see Table 7 caption.
ND: non-detectable.

Table 11. Volatile compounds of various commercial *Kapi* (Cont.)

Volatile compounds	Peak area (Abundance) × 10^5										
	S1[*]	S2	S3	S4	S5	S6	S7	S8	S9	S10	S11
2-Pentanone	ND	ND	ND	ND	760	ND	ND	ND	ND	ND	ND
2-heptanone	ND	1344	ND	ND	825	2788	ND	2302	4951	4330	ND
2-hexanone	893	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
2-Octanone	ND	361	ND	ND	ND	ND	ND	701	ND	1956	ND
3-Octanone	ND	ND	ND	449	1271	ND	ND	ND	ND	ND	ND
7-Octen-2-one	ND	ND	277	ND	271	ND	ND	ND	570	1105	ND
3,5- Octadiene-2-one	331	1578	645	ND	ND	ND	ND	ND	ND	ND	ND
Hydrocarbon											
1-phenylpropane	ND	256	ND	ND	ND	ND	ND	ND	ND	ND	ND
2,3-butanediene	ND	ND	2401	ND	ND	ND	ND	ND	ND	ND	ND
2,6-cyclohexadien	1165	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
3- dodecyne	197	614	454	ND	314	ND	279	229	207	341	ND
3-Tetradocene	163	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
Cyclododecane	905	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
Others											
Propaonic acid	583	ND	ND	ND	ND	ND	ND	ND	846	ND	ND
Butanic acid	ND	ND	ND	ND	ND	ND	ND	1948	ND	ND	ND
Pentanoic acid	358	ND	ND	1360	317	687	461	ND	2507	2349	938
Benzoic acid	ND	ND	ND	ND	736	ND	ND	ND	ND	ND	ND
Phenol	5625	10845	14174	8337	2592	4969	10687	2061	7516	693	31179
Indole	696	973	1597	345	269	772	344	163	622	1852	ND

[*]Samples: see Table 7 caption.

ND: non-detectable.

Abundance of aldehydes e.g. pentanal, hexanal, etc. in *Kapi* was quite low. It was noted that benzaldehyde, with a pleasant almond, nutty and fruity aroma (Vejaphan *et al.,* 1988) was found in all samples. Aldehydes were more likely generated from lipid oxidation during fermentation. Branched short chain aldehydes or aromatic aldehydes plausibly resulted from deamination of amino acids (Steinhaus and Schieberle, 2007). Groot and Bont (1998) noted that some bacteria had aminotransferase in cell extract, which converted phenylalanine into phenylpyruvic acid. Keto acid was further transformed to benzaldehyde. Takeungwongtrakul *et al.* (2012) reported that shrimp contained high amounts of ω-3 fatty acids, which were highly susceptible to lipid oxidation. Most of alkanals and alkenals were known to contribute to slightly rancid odors (Vejaphan *et al.,* 1988). However, *Kapi* had low fat content (1.41-3.67%) (Pongsetkul *et al.,* 2014). Moreover, Ho *et al.* (1989) reported that some aldehydes with unpleasant odors, may act as the important precursor of heterocyclic compounds.

Ketones were notably low in *Kapi*. Ketones found in *Kapi* included 2 pentanone, 2-heptanone, etc. Ketones seem to be responsible for the cheesy note in fish sauce odor (Peralta *et al.,* 1996). However, such compounds with low concentrations and high odor threshold values might not contribute to flavor of *Kapi* (Cha and Cadwallader, 1995).

Additionally, all samples contained phenol, but varied in abundance. Among the phenolic or aromatic compounds, toluene was more abundant in shrimp pastes, while phenol was more abundant in fish pastes (Vejaphan *et al.,* 1988). Toluene and phenol were reported to give an undesirable aroma in seafoods (Vejaphan *et al.,* 1988). S11 (*Kapi* Samut Songkram) and S7 (*Kapi* Songkhla2) had the higher abundance in phenol than other samples.

All samples, except S11 (*Kapi* Samut Songkram), consisted of indole. The highest indole was found in S3 (*Kapi* Ranong2) sample. Indole is the degradation product from tryptophan and has been used as the index for shrimp spoilage (Chang *et al.,* 1983). The result indicated that the raw material might be varied in freshness and the decomposition of tryptophan during fermentation was different among samples. Overall, the abundance of the lipid-derived compounds was low in *Kapi*. Nitrogen-containing compounds, especially pyrazine, were probably the potent contributors to

odors and flavors of salted shrimp paste. Different volatile compounds in different samples more likely affected their sensory properties.

3.5.3 Sensory properties

Likeness score of different salted shrimp pastes is shown in Table 12. Generally, S9 (*Kapi* Rayong) had the highest likeness score for all sensory characteristics including appearance, color, odor, texture, flavor and overall ($p < 0.05$). However, based on overall likeness, S9 (*Kapi* Rayong) showed similar score with S1 (*Kapi* Satun), S2 (*Kapi* Ranong1), S4 (*Kapi* Krabi1), S5 (*Kapi* Krabi2), S6 (*Kapi* Songkhla1), S8 (*Kapi* Samut Sakorn) and S10 (*Kapi* Chachoengsao) ($p > 0.05$). S11 (*Kapi* Samut Songkram) generally had the lowest score ($p < 0.05$) but there was no difference in overall likeness in comparison with S3 (*Kapi* Ranong2) and S7 (*Kapi* Songkhla2) ($p > 0.05$). The differences in sensorial characteristics among samples could be influenced by the differences in raw material used, ingredients, fermentation process and conditions (Beriain *et al.,* 2000). Therefore, it was likely that chemical compositions and physical properties contributed to the varied likeness of different salted shrimp pastes. In the present study, taste or flavor mainly affected the sensory quality (overall-liking) of foods. Different tastes or flavors were possibly caused by differences in amino acid composition (Table 10) and volatile compounds (Table 11). S9 (*Kapi* Rayong), which had the highest likeness score, contained the highest total amino acid content (68.95 mg/g sample). It contained high Glu/Gln (12.08 mg/g sample) and Asp/Asn (7.1 mg/g sample). Kim *et al.* (2005) reported that taste of *Kapi* was influenced by Glu and Asp, affecting umami and sweetness, respectively. For color likeness, S1 (*Kapi* Satun), S3 (*Kapi* Ranong2), S6 (*Kapi* Songkhla1), S8 (*Kapi* Samut Sakorn) and S11 (*Kapi* Samut Songkram) showed the lowest score ($p < 0.05$). S3 (*Kapi* Ranong2) and S11 (*Kapi* Samut Songkram) had the lowest texture likeness score ($p < 0.05$). This was more likely due to the differences in processes, ingredients as well as individual perception.

Table 12. Likeness score of various commercial *Kapi*

Attributes	S1[*]	S2	S3	S4	S5	S6	S7	S8	S9	S10	S11
Appearance	7.30 ± 1.59^{bc}	7.60 ± 1.39^{ab}	7.25 ± 1.07^{bc}	7.85 ± 1.31^{ab}	7.55 ± 1.28^{ab}	6.90 ± 1.25^{b}	6.95 ± 1.79^{bc}	7.10 ± 1.52^{bc}	8.30 ± 0.92^{a}	7.85 ± 1.04^{ab}	6.45 ± 1.76^{c}
Color	7.15 ± 1.46^{c}	7.55 ± 1.39^{ab}	6.95 ± 1.32^{c}	7.65 ± 1.18^{ab}	7.55 ± 1.15^{ab}	6.75 ± 1.07^{c}	7.20 ± 1.85^{ab}	6.75 ± 1.86^{c}	8.15 ± 0.81^{a}	7.65 ± 1.18^{ab}	6.95 ± 1.64^{c}
Odor	7.30 ± 1.42^{ab}	6.50 ± 1.79^{bc}	5.45 ± 1.43^{cd}	6.65 ± 1.79^{ab}	7.40 ± 1.57^{ab}	6.95 ± 1.85^{ab}	5.05 ± 1.82^{d}	7.00 ± 1.92^{ab}	7.80 ± 1.64^{a}	6.85 ± 1.76^{ab}	4.90 ± 2.02^{d}
Texture	7.20 ± 1.54^{b}	7.25 ± 1.33^{b}	6.10 ± 1.41^{c}	7.80 ± 1.47^{ab}	7.35 ± 1.63^{ab}	7.55 ± 1.57^{ab}	6.10 ± 1.25^{c}	7.75 ± 1.16^{ab}	8.35 ± 0.99^{a}	8.00 ± 0.92^{ab}	5.65 ± 2.23^{c}
Flavor	7.50 ± 1.85^{ab}	7.50 ± 1.67^{ab}	6.05 ± 1.67^{cd}	6.95 ± 1.76^{bc}	7.40 ± 1.35^{ab}	7.40 ± 1.43^{ab}	6.25 ± 1.55^{cd}	7.40 ± 1.35^{ab}	8.15 ± 1.18^{a}	7.70 ± 1.13^{ab}	5.60 ± 1.67^{d}
Overall	7.25 ± 1.83^{ab}	7.35 ± 1.76^{a}	6.25 ± 1.52^{bc}	7.30 ± 1.53^{ab}	7.30 ± 1.30^{ab}	7.25 ± 1.45^{ab}	6.20 ± 1.51^{c}	7.40 ± 1.35^{a}	8.30 ± 1.08^{a}	7.55 ± 1.28^{a}	5.80 ± 2.04^{c}

[*]Samples: see Table 7 caption.

Score are based on a 9-point hedonic scale (1: Dislike extremely, 5: Neither like nor dislike, 9: Like extremely).

Values are given as mean ± SD (n = 3). Different lowercase superscripts within the same row indicate the significant differences ($p < 0.05$).

3.5.4 Browning and Maillard reaction product

3.5.4.1 UV-absorbance and browning intensity

UV-absorbance (A_{280} and A_{295}) of water extract of different *Kapi* samples is shown in Table 13. The different extracts had varying UV-absorbance ($p < 0.05$). A_{280} and A_{295} have been used to determine the formation of non-fluorescent intermediate compounds of the Maillard reaction (Ajandouz *et al.,* 2001). Among all samples, water extract of S7 (*Kapi* Songkhla2) had the highest A_{280} and A_{295}. The higher A_{280} and A_{295} suggested the higher formation of an uncolored compound, which could be the precursor of the Maillard reaction (Benjakul *et al.,* 2005).

Table 13. A_{280}, A_{295}, browning intensity (A_{420}) and fluorescence intensity of water extracts from various commercial *Kapi*

Samples*	A_{280}	A_{295}	Browning intensity (A_{420})	Fluorescence intensity
S1	1.31 ± 0.00^{c}	0.97 ± 0.04^{d}	0.26 ± 0.00^{b}	665.27 ± 4.31^{abc}
S2	1.19 ± 0.05^{d}	0.52 ± 0.00^{i}	0.28 ± 0.00^{a}	683.72 ± 21.99^{a}
S3	0.43 ± 0.00^{h}	0.64 ± 0.00^{f}	0.25 ± 0.00^{c}	665.00 ± 3.67^{abc}
S4	1.10 ± 0.02^{e}	0.86 ± 0.00^{e}	0.27 ± 0.00^{a}	671.80 ± 1.11^{ab}
S5	1.67 ± 0.03^{b}	1.01 ± 0.01^{c}	0.24 ± 0.00^{e}	649.67 ± 2.65^{c}
S6	1.07 ± 0.01^{e}	0.54 ± 0.00^{hi}	0.20 ± 0.00^{h}	603.78 ± 13.72^{e}
S7	1.73 ± 0.01^{a}	1.28 ± 0.00^{a}	0.26 ± 0.00^{c}	662.37 ± 6.71^{bc}
S8	1.70 ± 0.01^{ab}	1.06 ± 0.05^{b}	0.25 ± 0.00^{d}	662.31 ± 10.38^{bc}
S9	1.08 ± 0.04^{e}	0.59 ± 0.01^{g}	0.21 ± 0.00^{g}	629.21 ± 7.69^{d}
S10	1.00 ± 0.01^{f}	0.57 ± 0.01^{gh}	0.22 ± 0.00^{f}	625.81 ± 13.99^{d}
S11	0.90 ± 0.01^{g}	0.47 ± 0.01^{j}	0.25 ± 0.01^{c}	670.71 ± 9.80^{ab}

*Sample: see Table 7 caption.
The sample was 5-fold diluted prior to measurement.
Values are given as mean ± SD (n=3). Different lowercase superscripts in the same column indicate the significant difference ($p < 0.05$).

Browning intensity (A_{420}) of all samples is shown in Table 13. The different *Kapi* samples had different browning intensity ($p < 0.05$). Water extract of S2 (*Kapi* Ranong1) showed the highest A_{420}, whereas S6 (*Kapi* Songkhla1) had the lowest A_{420} ($p < 0.05$). Generally, the higher A_{420} indicated higher browning development in the final stage of the Maillard reaction (Ajandouz *et al.,* 2001; Morales and Jimenez-

Perez, 2001). Therefore, the differences in browning intensity were more likely affected by raw material, ingredient and process used, which could vary from place to place.

3.5.4.2 Fluorescence intensity

Fluorescence intensity of water extracts from *Kapi* is shown in Table 13. Among all samples, water extract of S2 (*Kapi* Ranong1) had the highest fluorescence intensity, whereas that of S6 (*Kapi* Songkhla1) had the lowest intensity ($p < 0.05$). The results of fluorescence intensity were in accordance with those of browning intensity (Table 13). The relationship between browning intensity and fluorescence intensity suggested that a large proportion of fluorescent intermediate product was converted into a brown polymer. Jing and Kitts (2002) reported that the development of fluorescent compounds occurred in the Maillard reaction prior to the generation of brown pigments. Fluorescent compounds are possible precursors of brown pigments (Labuza and Baisier, 1992). Therefore, the lower fluorescence intensity was presumably due to the lower precursor for browning reaction. Generally, both non-fluorescent and fluorescent intermediates are formed and turn into brown pigments in the Maillard reaction (Morales *et al.,* 1996). The difference in fluorescence intensity and UV-absorbance of samples suggested that different types of intermediate products, either fluorescent or non-fluorescent compound, were formed and underwent the final stage of reaction at different rates (Benjakul *et al.,* 2005). However, the fluorescent intermediate was more reactive in formation of brown color than non-fluorescent compounds (Benjakul *et al.,* 2005). The browning development could affect the color and acceptability of salted shrimp paste differently.

3.5.5 Antioxidative activities

3.5.5.1 DPPH radical scavenging activity

The antioxidative activities of the water extracts of different *Kapi* are shown in Table 14. Water extract of S1 (*Kapi* Satun) showed the highest DPPH radical scavenging, whereas that of S6 (*Kapi* Songkhla1) had the lowest DPPH radical scavenging ($p < 0.05$). All samples had the ability to quench DPPH radicals. The DPPH radical had an absorbance at 515-520 nm. The color changed from purple to yellow by

acceptance of a hydrogen radical and it became a stable diamagnetic molecule (Benjakul *et al.,* 2009). This indicated that peptides or free amino acids in the salted shrimp paste possessed the ability to donate the hydrogen atom to free radicals, in which the propagation process could be retarded (Faithong *et al.,* 2010). Water extract of all samples had DPPH radical scavenging in the range of 1.12-8.99 µmol TE/g sample. Antioxidant peptides in salted shrimp paste were more likely water soluble peptides. Furthermore, other antioxidative compounds including MRPs were also present in salted shrimp paste. Those peptides or MRPs were mostly hydrophilic in nature and were extracted into water effectively (Binsan *et al.,* 2008).

3.5.5.2 ABTS radical scavenging activity

Water extracts from different *Kapi* showed different ABTS radical scavenging capacities (Table 14). ABTS assay is an excellent tool for determining the antioxidant activity of hydrogen-donating antioxidants (scavengers of aqueous phase radicals) and of chain breaking antioxidants (scavenger of lipid peroxyl radicals) (Leong and Shui, 2002). This method can determine both hydrophilic and lipophilic antioxidants (Sun and Tanumihardjo, 2007). ABTS radical-scavenging activities of water extracts were generally similar to those observed for DPPH radical-scavenging activity. Water extract of S1 (*Kapi* Satun) showed the highest ABTS radical scavenging capacity (17.87 µmol TE/g sample), whereas that of S6 (*Kapi* Songkhla1) had the lowest ABTS radical scavenging capacity (13.04 µmol TE/g sample) ($p < 0.05$). The result suggested that water soluble fractions from salted shrimp paste might scavenge $ABTS^{\bullet}$, mainly by hydrophilic antioxidants.

Table 14. Antioxidative properties of water extract from various commercial *Kapi*

Samples*	DPPH radical scavenging activity (μmol TE/g sample)	ABTS radical Scavenging activity (μmol TE/g sample)	FRAP (μmol TE/g sample)	Chelating activity (μmol EE/g sample)	H_2O_2 radical scavenging activity (μmol TE/g sample)	Singlet oxygen scavenging activity (μmol TE/g sample)
S1	8.99±0.58[a]	17.87±0.33[a]	26.97±0.09[a]	16.54±1.46[a]	38.48±2.18[a]	76.96±2.02[a]
S2	2.83±0.11[d]	15.65±0.02[d]	13.86±0.27[de]	8.86±0.29[fg]	32.54±0.57[de]	34.23±2.78[de]
S3	2.35±0.11[d]	13.67±0.45[e]	14.47±0.07[de]	9.15±0.43[ef]	31.56±0.54[ef]	53.62±1.15[b]
S4	2.71±0.57[d]	16.62±0.58[c]	17.51±0.85[bc]	11.39±0.42[d]	30.34±1.94[ef]	76.23±5.17[a]
S5	3.65±0.14[c]	13.91±0.04[e]	17.84±0.50[bc]	12.26±0.23[cd]	32.00±0.27[def]	71.69±2.73[a]
S6	1.12±0.02[e]	13.04±0.08[f]	12.70±0.34[e]	7.86±0.31[g]	30.12±1.77[f]	8.57±1.68[e]
S7	8.28±0.09[b]	17.24±0.23[b]	19.80±0.18[b]	13.75±0.51[b]	38.61±0.16[a]	76.17±3.24[a]
S8	3.51±0.28[c]	16.73±0.50[bc]	19.33±1.27[b]	13.31±0.20[bc]	34.17±2.05[cd]	56.09±0.37[b]
S9	3.75±0.03[c]	16.67±0.13[c]	15.65±0.21[cd]	8.85±0.42[fg]	35.92±0.20[bc]	21.58±3.62[de]
S10	2.78±0.08[d]	15.50±0.24[d]	15.87±3.91[cd]	10.20±0.75[e]	36.92±0.09[ab]	29.51±3.16[cd]
S11	3.36±0.42[c]	16.23±0.11[c]	16.29±1.58[cd]	12.02±1.01[d]	36.39±0.26[ab]	40.67±5.54[c]

*Samples: see Table 7 caption.

Values are given as mean ± SD (n=3). Different lowercase superscripts in the same column indicate the significant difference ($p < 0.05$).

3.5.5.3 Ferric reducing antioxidant power (FRAP)

FRAP is generally used to measure the capacity of a substance in reducing TPTZ-Fe(III) complex to TPTZ-Fe(II) complex (Benzie and Strain, 1996; Kittipattanabawon *et al.,* 2012). Varying FRAP was found among water extracts from different samples (Table 14), suggesting different capability of providing the electron. Among all samples, water extract from S1 (*Kapi* Satun) showed the highest FRAP, whereas that of S6 (*Kapi* Songkhla1) had the lowest FRAP ($p < 0.05$). This result was in agreement with DPPH and ABTS radical scavenging activities. Generally, low molecular weight peptides and amino acids have been reported to possess antioxidant activity (Binsan *et al.,* 2008; Benjakul *et al.,* 2009; Faithong *et al.,* 2010; Kittipattanabawon *et al.,* 2012). It has been reported that commercially available *Kapi*, traditional shrimp paste in Thailand, showed antioxidant activities including DPPH, ABTS radical scavenging activity and FRAP (Faithong *et al.,* 2010). Hydrolysis of proteins or peptides was progressed throughout the prolonged fermentation. Those free amino acids or peptides might undergo Maillard reaction, in which the resulting products possessed antioxidative activity (Lertittikul *et al.,* 2007).

3.5.5.4 Metal chelating activity

Metal chelating activity of water extracts from different *Kapi* is shown in Table 14. Transition metal irons catalyze the generation of reactive oxygen species, including hydroxyl radical ($^{\bullet}OH$) and superoxide radical ($O_2^{\bullet -}$), leading to oxidation of unsaturated lipids and promoting oxidative damage at different levels (Saiga *et al.,* 2003; Carrasco-Castilla *et al.,* 2012). Water extract from S1 (*Kapi* Satun) showed the highest metal chelating activity (16.54 μmol EE/g sample) and the lowest activity was found in the extract from S6 (*Kapi* Songkhla1) (7.86 μmol EE/g sample) ($p < 0.05$). Among all samples, the different iron chelating activity might be related to the differences in amino acid composition of peptides. It has been reported that chelation of iron was also associated with Asp/Asn, Glu/Gln, His, and Cys contents (Carrasco-Castilla *et al.,* 2012). Asp and Glu might be responsible for iron chelation (Xia *et al.,* 2008; Peng *et al.,* 2010; Carrasco-Castilla *et al.,* 2012). However, chelating activity of

peptides also depends on other factors such as peptide structure, steric effects and molecular weight (Carrasco-Castilla *et al.,* 2012).

3.5.5.5 H_2O_2 radical and singlet oxygen scavenging activity

Capacity of scavenging of hydrogen peroxide (H_2O_2) and singlet oxygen ($^1O_2$) of water extracts from different *Kapi* is presented in Table 14. Hydrogen peroxide and singlet oxygen as reactive oxygen species (ROS) can cause oxidative stress and damage of biomolecule in the cell, leading to cell death and serious chronic diseases (Suh *et al.,* 2011). Hydrogen peroxide, which is a weak oxidizing agent, is not directly involved in the initiation of lipid oxidation because its reduction potential is lower than that of unsaturated fatty acids (Choe and Min, 2005; Kittipattanabawon *et al.,* 2012). However, hydrogen peroxide can be implicated indirectly in lipid oxidation (Intarasirisawat *et al.,* 2013). Furthermore, hydrogen peroxide is a reactive non radical, which can permeate biological membranes and be converted to more reactive species such as hydroxyl radical and singlet oxygen (Choe and Min, 2005; Intarasirisawat *et al.,* 2013).

Among water extracts of all samples, H_2O_2 radical scavenging activity varied from 30.12 to 38.61 μmol TE/g sample and singlet oxygen scavenging activity was in range of 8.57-76.96 μmol TE/g sample. Kittiphattanabawon *et al.* (2012) suggested that peptides with the shorter chain length might be able to trap or bind with singlet oxygen to a higher extent. Singlet oxygen, which is a highly reactive, electrophilic and non-radical molecule, can be formed by the reaction between photosensitizers and triple oxygen in the presence of light. Singlet oxygen had low activation energy and its reaction rate with foods is much greater than that of triplet oxygen (Min and Boff, 2002). Singlet oxygen can directly react with electron-rich double bonds of unsaturated fatty acids without the formation of free-radical intermediates (Choe and Min, 2005). Composition and sequence of amino acid, structure of peptide, and the solvent accessibility of the amino acids in the peptide had the impact on antioxidative activity of peptides (Lertittikul *et al.,* 2007; Binsan *et al.,* 2008). Therefore, different salted shrimp pastes showed varying antioxidative activities, most likely associated with varying peptides and MRPs.

3.6 Conclusion

Different *Kapi* had varying amino acid compositions. Glu and Asp were the major amino acids, which might contribute significantly to the taste and flavor of *Kapi.* Volatile compounds in samples were different in abundance. Pyrazine derivatives were the major volatile components in *Kapi.* Water extract contained intermediate and final products of Maillard reaction. All samples possessed antioxidant activity, which could be an important source of natural antioxidants.

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CHAPTER 4

CHARACTERIZATION OF ENDOGENOUS PROTEASE AND THE CHANGES IN PROTEOLYTIC ACTIVITY OF *ACETES VULGARIS* AND *MACROBRACHIUM LANCHESTERI* DURING *KAPI* PRODUCTION

4.1 Abstract

Characteristics of endogenous proteases of shrimp, *Acetes vulgaris* (AP) and *Macrobrachium lanchesteri* (MP) as well as the changes in proteolytic activity during *Kapi* production were investigated. Maximal activity of AP and MP was found at pH 7, 60°C and pH 8, 60°C, respectively. Activity of both proteases decreased with increasing NaCl concentration (0-30%). Both extracts were strongly inhibited by *N*-ethylmaleimide-phenylmethane-sulfonyl fluoride (PMSF) and soybean trypsin inhibitor (SBTI), suggesting that major proteases belonged to serine proteases. This was coincidental with high trypsin activity toward BAPNA and chymotrypsin activity toward BTEE. Proteolytic, trypsin and chymotrypsin activities were detectable throughout *Kapi* fermentation. The activity was decreased when salting was implemented. Nevertheless, activities increased continuously with increasing fermentation time. During *Kapi* production, proteins underwent degradation as indicated by the formation of oligopeptides and disappearance of myosin heavy chain and actin. Therefore, both endogenous and microbial proteases were more likely involved in proteolysis of shrimp during *Kapi* production.

4.2 Introduction

Kapi is a traditional salted shrimp paste of Thailand. It is mainly produced from the marine shrimp or krill, in which salt is added at a shrimp/salt ratio of 5:1 (w/w) (Faithong and Benjakul, 2012). During production, the moisture content of mixture is reduced by sun drying, and then thoroughly blended or homogenized to produce semisolid paste. The paste is fermented for at least 1 month or until the desired

flavor is developed (Pongsetkul *et al.,* 2014). *Kapi* is usually used as a condiment to enhance the palatability of foods (Peralta *et al.,* 2008).

In general, krill (*Mesopodopsis orientalis*) is the traditional raw material for *Kapi* making. However, krill stocks have dropped by 3% per year since 1990 (Meland and Willassen, 2007). Therefore, the alternative source must be taken into consideration. Small shrimp belonging to genus *Acetes* have been recently used to produce shrimp paste, sauce and other fermented products in Southeast Asian countries. Among, *Acetes, A. vulgaris* is becoming one of the popular species commonly used for *Kapi* production in the southern part of Thailand, owing to its availability (Faithong and Benjakul, 2012; Pongsetkul *et al.,* 2014). Recently, because of its abundance and lower price, another species, *Macrobrachium lanchesteri*, a by-catch from commercial fishing, usually found in the southern part of Thailand throughout the year, has also become the new alternative raw material for *Kapi* production (Pongsetkul *et al.,* 2015). However, different appearance, flavor, taste as well as other sensorial properties were obtained in *Kapi* produced from both species (Pongsetkul *et al.,* 2014).

Proteolysis is an important biochemical change occurring during the fermentation of *Kapi.* During fermentation, protein hydrolysis is induced by endogenous proteases in shrimp/krill, used as raw material, as well as proteases produced by halophilic bacteria (Gildberg and Stenberg, 2001). Those proteases influence both texture and flavor development associated with several low molecular weight compounds, including peptides, amino acids, aldehydes, organic acids and amines (Pongsetkul *et al.,* 2014). Breakdown products of proteolysis more likely contribute to the characteristic flavor of fermented shrimp paste (Fadda *et al.,* 1998; Peralta *et al.,* 2008; Hajeb and Jinap, 2015). Thus, different raw material with varying amount and type of endogenous proteases might have the impact on final characteristics of *Kapi*. Nevertheless, no information regarding the biochemical properties of endogenous proteases in *A. vulgaris* and *M. lanchesteri* has been reported.

4.3 Objective

To characterize the endogenous proteases in *A. vulgaris* and *M. lanchesteri* and to monitor the changes in proteolytic activity during *Kapi* fermentation.

4.4 Materials and methods

4.4.1 Chemicals

Sodium caseinate, bovine hemoglobin, ethylenediaminetetraacetic acid (EDTA), pepstatin A, soybean trypsin inhibitor (SBTI), iodoacetic acid, *N-p*-tosyl-*L*-lysine chloromethyl ketone (TLCK), *N*-tosyl-*L*-phenyl-alanine chloromethyl ketone (TPCK), 1-(*L*-trans-epoxysuccinyl-leucylamino)-4-guanidinobutane (E-64), *N*-ethylmaleimide-phenylmethanesulfonyl fluoride (PMSF), iodoacetic acid, *N*-benzoyl-*L*-tyrosine ethyl ester (BTEE), *N*-*α*-benzoyl-*D*-*L*-arginine-p-nitroanilide (BAPNA) and malonaldehyde bis (dimethyl acetal) were purchased from Sigma Chemical Co. (St. Louis, MO). Potassium persulfate, acrylamide, *N,N,N',N'*-tetramethylethylenediamine and bis-acrylamide were procured from Fluka Chemical Co. (Buchs, Swizerland). Molecular weight markers were purchased from GE Healthcare UK, Limited (Buckinghamshire, U.K.). All chemicals were of analytical grade.

4.4.2 Sample collection

Live shrimp, *A. vulgaris* (average body length 15.6±1.4 mm, average wet weight 0.0413±0.0098 g, n=20) and *M. lanchesteri* (average body length 27.5±1.9 mm, average wet weight 0.0701±0.0107 g, n=20), were purchased from the village markets in Ko-yo and The-Pha in Songkhla province, Thailand, respectively. After capture, shrimp were transported in ice with a shrimp/ice ratio of 1:2 (w/w) in a polystyrene container to the Department of Food Technology, Prince of Songkla University, Hat Yai, Thailand, within approximately 2 h.

4.4.3 Characterization of protease in shrimp

4.4.3.1 Preparation of crude protease extract

Shrimp were powderized using liquid nitrogen and blender as described by Kittipatanabawon *et al.* (2012) and referred to as "Shrimp powder." Crude protease extract was prepared by the method of Klomklao *et al.* (2008). Shrimp powder (20 g) was suspended in 200 ml of 0.02 M sodium phosphate buffer (pH 8) containing 10 mM $CaCl_2$. The mixture was homogenized for 2 min using an IKA Labortechnik homogenizer (Selangor, Malaysia) at a speed of 11,000 rpm. The homogenate was stirred continuously at 4°C for 3 h, followed by centrifugation at 8,000×*g* for 30 min using a refrigerated centrifuge (Beckman Coulter, Avanti J-E Centrifuge, Palo Alto, CA). All procedures were carried out at 4°C. The supernatant was collected and kept at -40°C until used. Proteases in crude extracts from *A. vulgaris* and from *M. lanchesteri* were referred to as AP and MP, respectively.

4.4.3.2 Characterization of AP and MP

- Enzyme assay

Protease activities of AP and MP were assayed using hemoglobin (pH 2-6) and casein (pH 7-11) as substrates, according to the method of Klomklao *et al.* (2004). Activity was determined according to the TCA-Lowry assay. Crude extract (200 µl) was added into assay mixtures containing 2 mg of substrate, 200 µl of distilled water and 625 µl of reaction buffer. The mixture was incubated at the test pH and temperature for precisely 20 min. Reaction was terminated by adding 200 µl of 50% cold-TCA (w/v). Unhydrolyzed protein substrate was allowed to precipitate for 1 h at 4°C, followed by centrifuging at 10,000×*g* for 10 min. The oligopeptide content in the supernatant was determined by the Lowry assay (Lowry *et al.,* 1951) using tyrosine as a standard. A blank was run in the same manner, except the crude extract was added after addition of cold 50% TCA (w/v) (0-2°C). The activity (1 unit) was defined as the enzyme causing the release of 1 mmol as tyrosine equivalent/min/ml of crude extract.

- pH and temperature profile

Protease activities of AP and MP were assayed over the pH range of 2-11 (McIlvaine's buffer for pH 2-7 and 0.1 M glycine-NaOH for pH 8-11) containing 10 mM $CaCl_2$ as the activator of protease, particularly trypsin, at 60°C for 20 min. For the temperature profile study, the activity was determined at different temperatures (30, 40, 50, 55, 60, 65, 70 and 80°C), in a temperaturecontrolled water bath (Memmert, Germany), for 20 min at the optimal pH.

- Effect of NaCl concentration

The effect of NaCl on AP and MP was studied. NaCl was added into the reaction assay to obtain the final concentrations of 0, 5, 10, 15, 20, 25 and 30% (w/v). After addition of crude extract, the reaction was performed at the optimal pH, and 60°C for 20 min. The residual activity was determined using casein as the substrate.

- pH and Thermal Stability

The pH stability was evaluated by measuring the residual activity after incubation the crude extracts at various pHs at room temperature for 30 min. Different buffers used were mentioned above. For thermal stability, crude extract was subjected to heating at different temperatures (20, 30, 40, 50, 60, 70 and 80°C) for 30 min. Thereafter, the treated samples were suddenly cooled in iced water and then the remaining activity was determined.

- Inhibitor study

Effects of different protease inhibitors toward the proteolytic activity of AP and MP were determined according to the method of Sriket *et al.* (2011a). Crude extract was incubated with an equal volume of protease inhibitor solutions to obtain the designated final concentrations (1 mM pepstatin A, 0.1 mM E-64, 10 mM EDTA, 1 g/l SBTI, 5 mM PMSF, 5 mM TLCK, 5 mM TPCK and 1 mM Iodoacetic acid). The mixture was incubated at room temperature for 30 min. The remaining proteolytic

activity was determined at its optimal condition for 20 min. The percentage of inhibition was then calculated using the following formula:

$$\text{Inhibition (\%)} = 100 - \frac{A_s \times 100}{A_c}$$

where A_s and A_c are the proteolytic activity of sample treated with inhibitor and that of the control (sample without inhibitor), respectively. The control was conducted in the same manner except that deionized water was used instead of inhibitors.

4.4.4 Changes in proteolytic activity of shrimp during *Kapi* production

4.4.4.1 Preparation of *Kapi*

Kapi produced from *A. vulgaris* and *M. lanchesteri* were prepared. Live shrimp were mixed with salt at a shrimp/salt ratio of 5:1 (w/w), placed in the basket and covered with a cheese cloth. Salted shrimp were then left at room temperature overnight [S1]. The drained sample was mashed or pounded, followed by spreading out on fiberglass mats to dry with sunlight. The samples were restored in an earthen jar overnight and laid out again under the sun. This step was taken for 2-3 days until the samples were disintegrated and turned from pink to dark purplish brown and moisture content was in the range of 35-40% [S2]. Salted shrimp were transferred into earthen jars and covered with plastic bag tightly (close system). Then, samples were allowed to ferment for 1 month (30 days) at room temperature (28-30°C). During fermentation, the sample was taken at day 10[S3], 20[S4] and 30[S5] for determination of proteolytic activities.

4.4.4.2 Proteolytic activities

Crude extracts were prepared and the activities were determined under the optimal conditions using casein as a substrate as described above.

Trypsin activity of all samples was measured using BAPNA as a substrate according to the method of Senphan and Benjakul (2014). Sample (200 μl) was mixed with 200 μl ml of distilled water and 1,000 μl ml of reaction buffer (0.02 M sodium phosphate buffer, pH 8, containing 10 mM $CaCl_2$). The reaction was initiated

by adding 200 µl ml of 2 mg/ml BAPNA to the reaction mixture. After incubation for 20 min at 60°C, 200 ml of 30% acetic acid (v/v) were added to terminate the reaction. Production of *p*-nitroaniline was measured by monitoring the absorbance of reaction mixture at 410 nm. A blank was conducted in the same manner except that sample was added after addition of 30% acetic acid. One unit of trypsin activity was defined as the amount causing an increase of 0.01 in A410 per min.

Chymotrypsin activity was measured using BTEE as a substrate as per the method of Sriket *et al.* (2011b) with slight modifications. Sample (20 µl) was mixed with 3 ml of 1 mM BTEE in 0.02 M sodium phosphate buffer, pH 8.0, containing 10 mM $CaCl_2$. The reaction was conducted at 25°C for 20 min. Production of benzoyltyrosine was measured by monitoring the increase in absorbance at 256 nm. A blank was prepared in the same manner, but assay buffer was used instead of BTEE solution. One unit of chymotrypsin esterase activity was defined as the amount causing an increase of 0.01 in A_{256} per min. Both trypsin and chymotrypsin activities were reported as units/g dry weight sample.

4.4.4.3 Molecular weight determination and activity staining

- Preparation of ammonium sulfate fraction

Crude extracts were prepared as previously described and were subjected to 40-60% saturated ammonium sulfate precipitation, according to the method of Khantaphant and Benjakul (2010) with a slight modification. After the addition of ammonium sulfate, the mixture was stirred gradually at 4°C for 30 min. Thereafter, the mixture was centrifuged at 11,000×*g* for 30 min at 4°C and the pellet obtained was dissolved in the minimum volume of 50 mM Tris-HCl buffer, pH 8. The solution was dialyzed against 20 volumes of the extraction buffer overnight at 4°C with three changes of dialysis buffer. The dialysate was kept in ice and referred to as “ammonium sulfate fraction, ASF.”

- SDS-Polyacrylamide Gel Electrophoresis (SDS-PAGE)

Protein patterns of ASF were determined by SDS-PAGE using 4% stacking gel and 12% running gel according to the method of Laemmli (1970). Ground sample (3 g) was solubilized in 27 ml of 5% (w/v) SDS (85°C). The mixture was homogenized for 1 min at a speed of 13,000 rpm and incubated at 85°C for 1 h to dissolve total proteins. Samples (15 mg protein) determined by the Biuret method were loaded onto the gel and subjected to electrophoresis at a constant current of 15 mA per gel using a Mini-Pro-tean II unit (Bio-Rad Laboratories, Inc., Richmond, CA). After electrophoresis, the gels were stained with 0.05% (w/v) Coomassie Blue R-250 in 50% (v/v) methanol and 7.5% (v/v) acetic acid for 30 min. Finally, gels were destained with a mixture of 50% (v/v) methanol and 7.5% (v/v) acetic acid for 30 min and destained again with a mixture of 5% (v/v) methanol and 7.5% (v/v) acetic acid for 1 h. Low molecular weight protein markers (GE Healthcare UK Limited, Buckinghamshire, U.K.) were used to estimate the molecular weight of proteins.

- Activity staining

Activity staining was performed using the method of Garcia-Carreno *et al.* (1993). ASF was mixed with sample buffer as mentioned previously. However, the mixture was not boiled prior to loading onto the gel (4% stacking and 12% separating gel). After electrophoresis, the gel (3 mg protein each lane) was washed in 2.5% Triton X-100 at 4°C for 15 min to remove SDS and to renature the proteins. The gels were then washed again with distilled water. The gels were immersed in 100 ml of 50 mM Tris-HCl buffer, pH 8, containing 2% casein (w/v) for 1 h at 4°C with a gentle agitation. Thereafter, the gels were incubated at 60°C for 1 h with constant agitation to develop the activity zone. The gels were washed again with distilled water, fixed, stained and destained as described above. Protease band was indicated by the development of a clear zone on a blue background. The bands with protease activity were calculated for their molecular weights.

4.4.5 Changes in proteins during *Kapi* production

During *Kapi* fermentation, the samples were taken for analyses at day 10, 20 and 30. Samples after salting and drying were also subjected to SDS-PAGE as described previously. Additionally, TCA soluble peptide and free amino acid contents of samples was determined according to the method of Pongsetkul *et al.* (2015). Ground sample (3 g) was homogenized with 27 ml of cold 5% TCA using a homogenizer at a speed of 11,000 rpm for 1 min. The homogenate was kept in ice for 30 min and centrifuged at 5,000×*g* for 20 min at 4°C using a refrigerated centrifuge (Model RC-B Plus centrifuge Newtown, CT). Soluble oligopeptide content in the supernatant was measured according to the Lowry method and expressed as mmol tyrosine equivalent/g dry weight sample.

4.4.6 Statistical analysis

Completely randomized design (CRD) was used throughout the study. All experiments were run in triplicate. Data were subjected to analysis of variance (ANOVA), and mean comparisons were carried out by the Duncan's multiple range test. Independent T-test was performed for pair comparison (Steel *et al.,* 1980). Analysis were performed using SPSS statistic program (Version 10.0) (SPSS, 1.2, 1998). For PCA (Principal Component Analysis), the XLSTAT Software (XLSTAT, 2008, Addinsoft, New York, NY) was used.

4.5 Results and discussion

4.5.1 Characteristics of proteases from both shrimp

4.5.1.1 pH and temperature profiles

Proteolytic activities of AP and MP were determined over the pH ranges of 2-11 at 60°C as shown in Figure 3A. Maximal activities were found at pH 7 for AP and pH 8 for MP. The results indicated that neutral proteases were predominant in *A. vulgaris*, while *M. lanchesteri* had alkaline proteases as the dominant enzyme. The activities decreased when the pH were in the very acidic or alkaline pH ranges (p <

0.05). At very acidic or alkaline conditions, the increased repulsion, governed by positive and negative charge, respectively, might induce the conformational changes (Klomklao *et al.,* 2004). This phenomenon led to unfolding of enzymes associated with the loss in their activity. It was noted that higher proteolytic activity was observed in alkaline pH, compared to acidic pH for both AP and MP. Heat stable alkaline protease has been reported to be responsible for autolysis of fish muscle (Klomklao *et al.,* 2008). Sriket *et al.,* (2011b) reported that the optimal pH of crude proteases from fresh water prawn (*Macrobrachium rosenbergii*) was 7. Digestive proteases from mid gut glands of *Penaeus indicus* and lobster, *Nephrops norvegicus*, had the optimal pH of 7.7 and 8, respectively (Omondi and Stark, 2001). Buarque *et al.* (2009) reported that digestive protease from the mid gut gland of *Farfantepenaeus paulensis* showed the maximal activity at pH 8.

The temperature profiles of AP and MP are presented in Figure 3B. When activity was assayed at their optimum pH over temperature range of 30-80°C, the highest activity was observed at 60°C ($p < 0.05$) for both AP and MP. MP showed the higher proteolytic activity than AP when tested at the same temperature. A sharp decrease in activity was observed as the temperature increased, presumably as a result of thermal inactivation. At high temperature, enzyme molecule underwent unfolding, resulting in the loss in activity (Sriket *et al.,* 2011b). The optimal temperature of *A. vulgaris* and *M. lanchesteri* was similar to that of crude protease from fresh water prawn (*Macrobrachium rosenbergii*), but was different from that of crude protease from pink shrimp and shrimp (*Penaeus orientalis*), which had optimal temperatures of 70 and 45°C, respectively (Oh *et al.,* 2000: Buarque *et al.,* 2009).

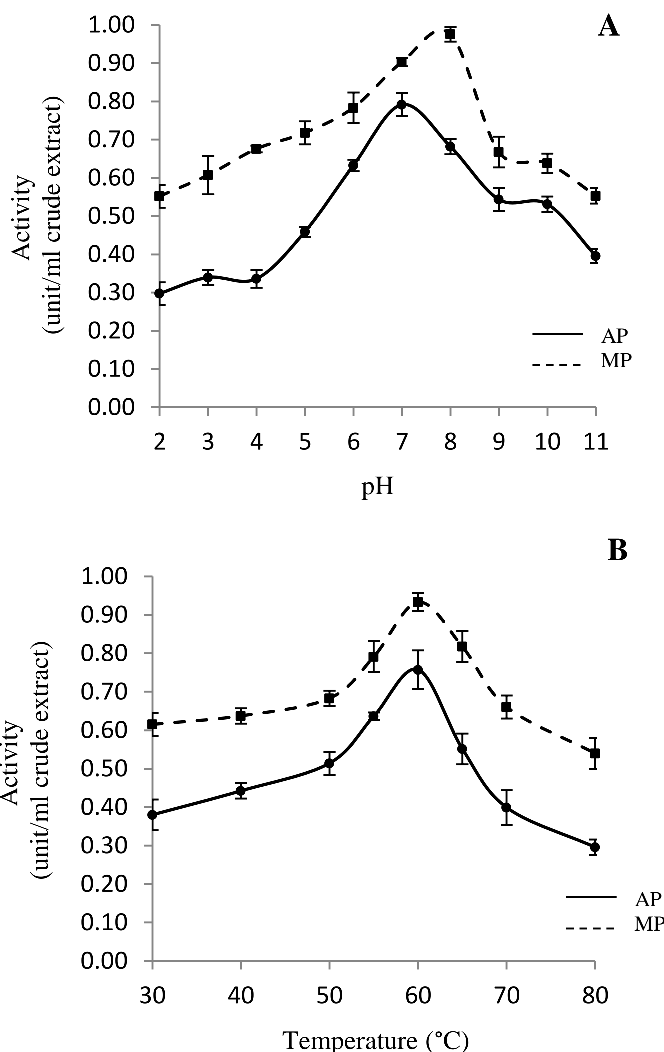

Figure 3. pH profile (A) and temperature profile (B) of crude proteases from *A. vulgaris* (AP) and *M. lanchesteri* (MP).

Bars represent the standard deviation from triplicate determinations.

4.5.1.2 pH and thermal stability

Both AP and MP had the similar pH stability over a pH range of 2-11 as shown in Figure 4A. Both AP and MP were quite stable at pH 6-8, with the remaining activity above 70%. The decrease in activity was observed when both extracts were exposed to very acidic or very alkaline pH conditions. The stability of enzyme at a particular pH might be governed by the net charge of the enzyme (Klomklao *et al.,* 2006). At extreme pHs, strong intramolecular electrostatic repulsion between negatively or positively charged domain might cause unfolding of enzyme molecules

(Klomklao *et al.,* 2009). The pH stability in alkaline pH range (9-11) was higher than acidic counterpart (2-5). Comparing with the activity at neutral pH (pH 7), the decrease more than 50% was found at pH values below 5. Thus, the inactivation was more pronounced at pH below 5. Endogenous proteases might undergo denaturation under the acidic conditions, where the conformational change took place. As a consequence, the proteases could not bind to the substrate properly. Inactivation at acidic pH is a common phenomenon for anionic trypsins (Sriket *et al.,* 2012).

Thermal stability of AP and MP is depicted in Figure 4B. Both AP and MP were stable when heated up to 60°C. The sharp loss in activity was noticeable after incubation at temperature above 60°C. When the enzyme was heated at sufficiently high temperature, its conformation could be altered. As a result, the lowered activity could be attained (Sriket *et al.,* 2012). At high temperatures, the enzyme more likely underwent denaturation and lost its activity. Kim *et al.* (1992) reported that trypsin activity of crayfish hepatopancreas was stable up to 40°C and the activity almost disappeared when heated at temperatures higher than 60°C. Bustos *et al.* (1999) reported that trypsin-like enzyme extracted from Antarctic krill (*Euphausia superba*) processing wastewater showed an unusually high stability, in which over 40% of the initial activity were retained after 60 days of storage at 45°C. These observations were opposite to the behavior of the pure enzymes, which were completely inactivated after 30 min at 45°C. Numerous disulfide linkages, as well as stronger hydrophobic interactions in the interior of the protein contribute to protein thermal stability (Vannabun *et al.,* 2014). Crude proteases of *A. vulgaris* and *M. lanchesteri* had slightly high optimum temperature (60°C) and high thermal stability. This might be due to the presence of disulfide linkages or hydrophobic interactions of those enzymes. Klomklao *et al.* (2009) reported that disulfide bonds may stabilize a folded conformation of trypsin, although fish and shellfish trypsins had a lower number of intramolecular disulphide bonds, compared with the mammal counterpart. Due to high thermal stability, those proteases in both shrimp might cause the substantial degradation of shrimp proteins during production of *Kapi*, performed at room temperature (28-35°C).

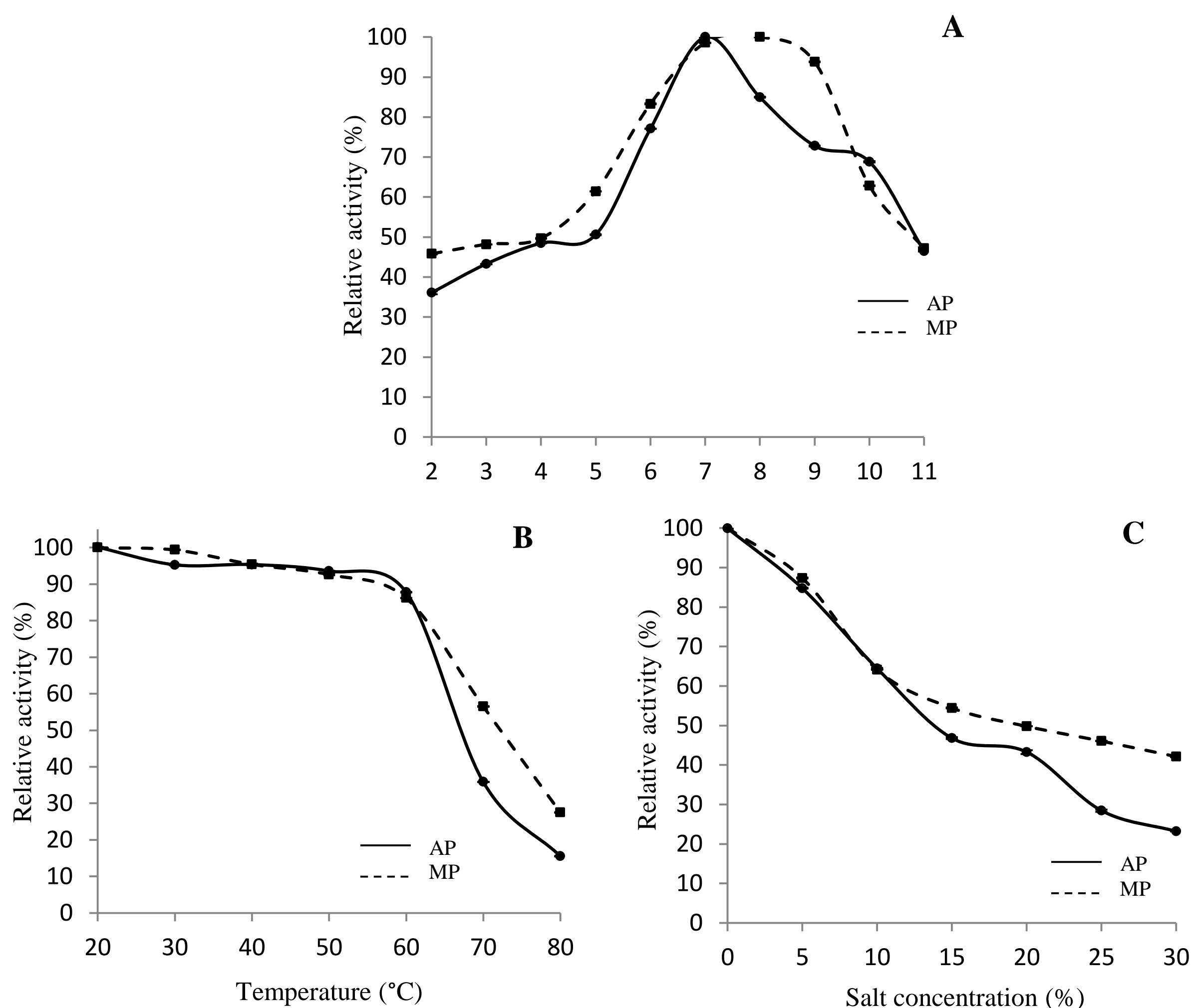

Figure 4. pH stability (A), thermal stability (B) and salt stability (C) of crude proteases from *A. vulgaris* (AP) and *M. lanchesteri* (MP).

Bars represent the standard deviation from triplicate determinations.

4.5.1.3 Effect of NaCl concentration

Proteolytic activity of AP and MP decreased gradually with increasing NaCl as depicted in Figure 4C. The decrease in activity might be due to the denaturation of proteases in the presence of high salt. The "salting out" effect was postulated to cause the enzyme denaturation. The water molecule is drawn from the protease molecule by salt, leading to the aggregation and denaturation of those enzymes (Klomklao *et al.,* 2004). The loss in activity more than 50% was noticeable when the concentration of NaCl was above 20% (w/v). Salting of shrimp overnight using 15-25% salt to inhibit

putrefactive microorganisms might lower autolysis mediated by endogenous proteases during *Kapi* production. Thus, different levels of salt used for making *Kapi* might affect the rate of protein degradation as well as some characteristics of final product.

4.5.1.4 Inhibitor study

The effect of various protease inhibitors on AP and MP is shown in Table 15. For AP, PMSF showed the highest inhibition (91.09%) ($p < 0.05$), followed by SBTI (50.29%), when tested at pH 7. For MP, the strongest inhibition by PMSF and SBTI (93.33 and 61.94%, respectively) was also observed ($p < 0.05$). Generally, SBTI and PMSF are serine protease inhibitors (Benjakul *et al.,* 2003). SBTI (Soybean trypsin inhibitor) is a single polypeptide that forms a stable stoichiometric enzymically inactive complex with trypsin, thereby reducing the availability of trypsin (Sriket *et al.,* 2012). It was noted that TLCK and TPCK, specific inhibitor of trypsin and chymotrypsin, respectively, also showed high inhibitory activity. It indicated that both trypsin and chymotrypsin were also present in both shrimp, mainly from digestive organs, particularly hepatopancreas. Other inhibitors, including Pepstatin A, E-64, EDTA and iodoacetic acid showed inhibition less than 50%.

Table 15. Effect of various inhibitors on activity of crude protease from *A. vulgaris* and *M. lanchesteri*

Inhibitors	Concentration	% Inhibition	
		AP	MP
Control	-	0	0
Pepstatin A	1 mM	39.06 ± 0.03^{d}	23.42 ± 0.08^{d}
E-64	0.1 mM	9.87 ± 0.06^{g}	7.48 ± 0.16^{g}
EDTA	10 mM	31.76 ± 0.02^{e}	15.14 ± 0.06^{e}
SBTI	1 g/L	50.29 ± 0.16^{b}	61.94 ± 0.22^{b}
PMSF	5 mM	91.09 ± 0.14^{a}	93.33 ± 0.09^{a}
TLCK	5 mM	46.11 ± 0.07^{c}	50.09 ± 0.15^{c}
TPCK	5 mM	48.52 ± 0.05^{bc}	48.75 ± 0.26^{c}
Iodoacetic acid	1 mM	19.71 ± 0.07^{f}	9.37 ± 0.08^{fg}

Crude protease extract (CPE) was mixed with the same volume of inhibitor for 30 min at room temperature. The residual activity was determined using casein as substrate at optimal condition for 20 min.

Mean ± SD from triplicate determinations. Different lowercase superscripts in the same column indicate the significant difference ($p < 0.05$).

Most shrimp including Caridean shrimp (*Crangon* spp.) (Teschke and Saborowski, 2004), *Metapenaeus ensis* (Hu and Leung, 2004), *Penaeus vannamei* (Garcia-Carreno *et al.,* 2008), *Macrobrachium rosenbergii* (Sriket *et al.,* 2012) and *Penaeus californiensis* (Navarrete-del-Toro *et al.,* 2015) contained serine proteases as the dominant enzymes. Furthermore, EDTA, which chelates the metal ions required for the enzyme, was able to partially lower proteolytic activity (31.76 and 25.14% inhibition for AP and MP, respectively). It has been reported that trypsin most likely required metal ions as cofactors for activity. Calcium ions were able to activate fish and crustacean trypsins (Klomklao *et al.,* 2009; Sriket *et al.,* 2012). From the inhibitor studies, and pH/temperature profiles, it can be inferred that crude protease from *A. vulgaris* and *M. lanchesteri* contained several groups of proteases, but serine proteases, both of trypsin and chymotrypsin-like enzymes, were prevalent.

4.5.2 Changes in proteolytic activity of shrimps during *Kapi* production

4.5.2.1 Proteolytic activities

Proteolytic activity of *A. vulgaris* and *M. lanchesteri* during *Kapi* production using casein as substrate is depicted in Figure 5. For fresh shrimp, proteolytic activity of AP was 3.52 units/g sample (dry weight basis). The activity was decreased to 2.00 units/g sample after being salted. The continuous increases after drying and during fermentation were obtained. The similar change of proteolytic activity was found for *M. lanchesteri* sample. However, the sample of *M. lanchesteri* showed the higher proteolytic activity at all steps tested, compared to that of *A. vulgaris* ($p < 0.05$). Slight decrease in proteolytic activity after salting might be due to the inactivation of endogenous proteases by salt. After drying of salted shrimp using sunlight, and fermenting in the close system, the proteolytic activity continuously increased in both *A. vulgaris* and *M. lanchesteri*. This might be result from the combination role of both remaining endogenous proteases in raw material used and proteases from halophillic bacteria produced during fermentation. During fermentation, not only endogenous protease in raw material but also microbial protease are involved in proteolysis. Therefore, proteolysis taken place in *Kapi* was mediated by both endogenous protease and microbial protease (Faithong and Benjakul, 2012). Several

halophilic bacteria with protease activity have been reported in *Kapi.* Those included *Oceanobacillus kapialis* (Namwong *et al.,* 2009), *Lentibacillus kapialis* (Pakdeeto *et al.,* 2007) and *Virgibacillus halodenitrificans* (Tanasupawat *et al.,* 2011), etc.

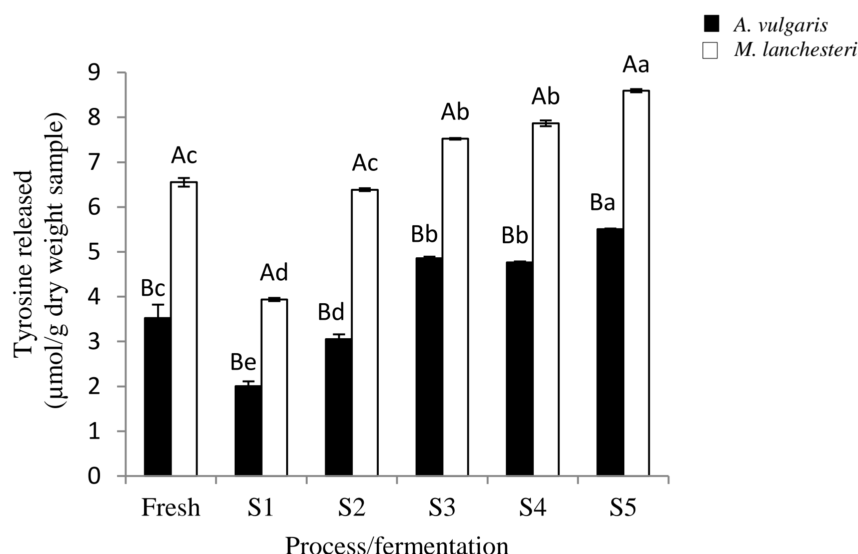

Figure 5. Proteolytic activity of crude proteases from *A. vulgaris* and *M. lanchesteri* during *Kapi* production. S1, sample after salting; 2, sample after drying; 3, 4, 5, sample during 10, 20 and 30 days of fermentation, respectively.

Bars represent the standard deviation from triplicate determinations. Different uppercase letters on the bars within the same step of production indicate the significant differences ($p < 0.05$). Different lowercase letters on the bars within the same species indicate the significant differences ($p < 0.05$).

Trypsin and chymotrypsin-like enzyme activities of *A. vulgaris* and *M. lanchesteri* during *Kapi* processing/fermentation are shown in Figure 6A and 6B, respectively. The result showed that both *A. vulgaris* and *M. lanchesteri* contained both trypsin-like and chymotrypsin-like proteases. This was in agreement with the inhibitor study, in which serine protease was dominant in both crude extracts (Table 15). Furthermore, trypsin and chymotrypsin were also detected, based on inhibitory activity of TPCK and TLCK (Table 15). For fresh shrimp, the trypsin-like enzyme activity of *A. vulgaris* was 4.37 unit/g dry sample, while the activity of 8.24 37 unit/g dry sample was found in *M. lanchesteri.* Similar to proteolytic activity toward casein, both trypsin and chymotrypsin like enzyme activities decreased slightly after salting. As the fermentation time increased, an increase in trypsin-like activity was observed up to the

end of final step (30 days of fermentation). Higher trypsin-like activity was observed in *M. lanchesteri* when compared to *A. vulgaris* at all steps. Similar changes in chymotrypsin-like enzyme activity were also found to those of trypsin-like enzyme. The result suggested that *M. lanchesteri* had the higher trypsin-like and chymotrypsin-like enzymes than *A. vulgaris* and these enzymes were still active during *Kapi* production. Those proteases more likely played a profound role in protein degradation, contributing to the quality of *Kapi.*

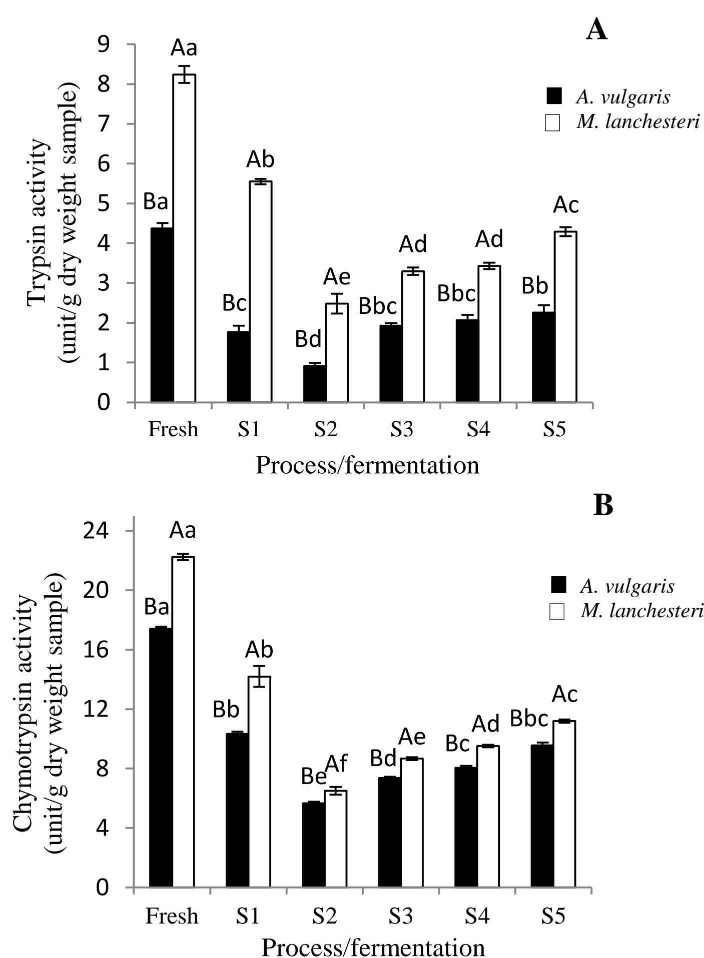

Figure 6. Trypsin activity (A) and chymotrypsin activity (B) of crude proteases from *A. vulgaris* and *M. lanchesteri* during *Kapi* production. S1, sample after salting; 2, sample after drying; 3, 4, 5, sample during 10, 20 and 30 days of fermentation, respectively.

Bars represent the standard deviation from triplicate determinations. Different uppercase letters on the bars within the same step of production indicate the significant differences ($p < 0.05$). Different lowercase letters on the bars within the same species indicate the significant differences ($p < 0.05$).

4.5.2.2 Protein pattern of crude proteases

Activity staining using casein as substrate of crude proteases extracted from *A. vulgaris* and *M. lanchesteri* during *Kapi* production is shown in Figure 7A and 7B, respectively. These activity bands with MWof 22, 20 and 15 kDa were found for AP from fresh samples. It was noted that activity band with MWof 22 kDa disappeared after salting. Three major bands of proteases with MW of 29, 20 and 15 kDa were observed after salting and during fermentation (Figure 7A). During fermentation, all three bands still remained up to 30 days. For MP, several bands of proteases with MW lower than 40 kDa were found. There were no changes in activity bands during processing and fermentation. Among all activity bands, those with MW of 36, 29, 22 and 20 kDa constituted as major proteases. Those proteases were found to have MW in the range of trypsin and chymotrypsin. Trypsin from digestive gland of *Penaeus japonicus* had the MW of 25 kDa (Galgani *et al.,* 1985). Trypsin from hepatopancreas of freshwater prawn (*Macrobrachium rosenbergii*) had a MW of 17 kDa (Sriket *et al.,* 2012). Wu *et al.* (2008) reported that MW of trypsin from North Pacific krill (*Euphausia pacifica*) was 33 kDa, while Sun *et al.* (2014) found that trypsin of this krill had MWof 26, 18 and 17 kDa. For chymotrypsin, the MW varied, depending on the species such as 27 kDa for *Penaeus monodon* (Tsai *et al.,* 1986), 33.2 kDa for *Penaeus vannamei* (Hernandez-Cortes *et al.,* 1997), 25 kDa for *Fenneropenaeus chinensis* (Shi *et al.,* 2008) and 35.7 for *Penaeus californiensis* (Navarrete-del-Toro *et al.,* 2015). The result was in accordance with inhibitor study, which indicated that serine protease (trypsin and chymotrypsin) were present in both AP and MP (Table 15).

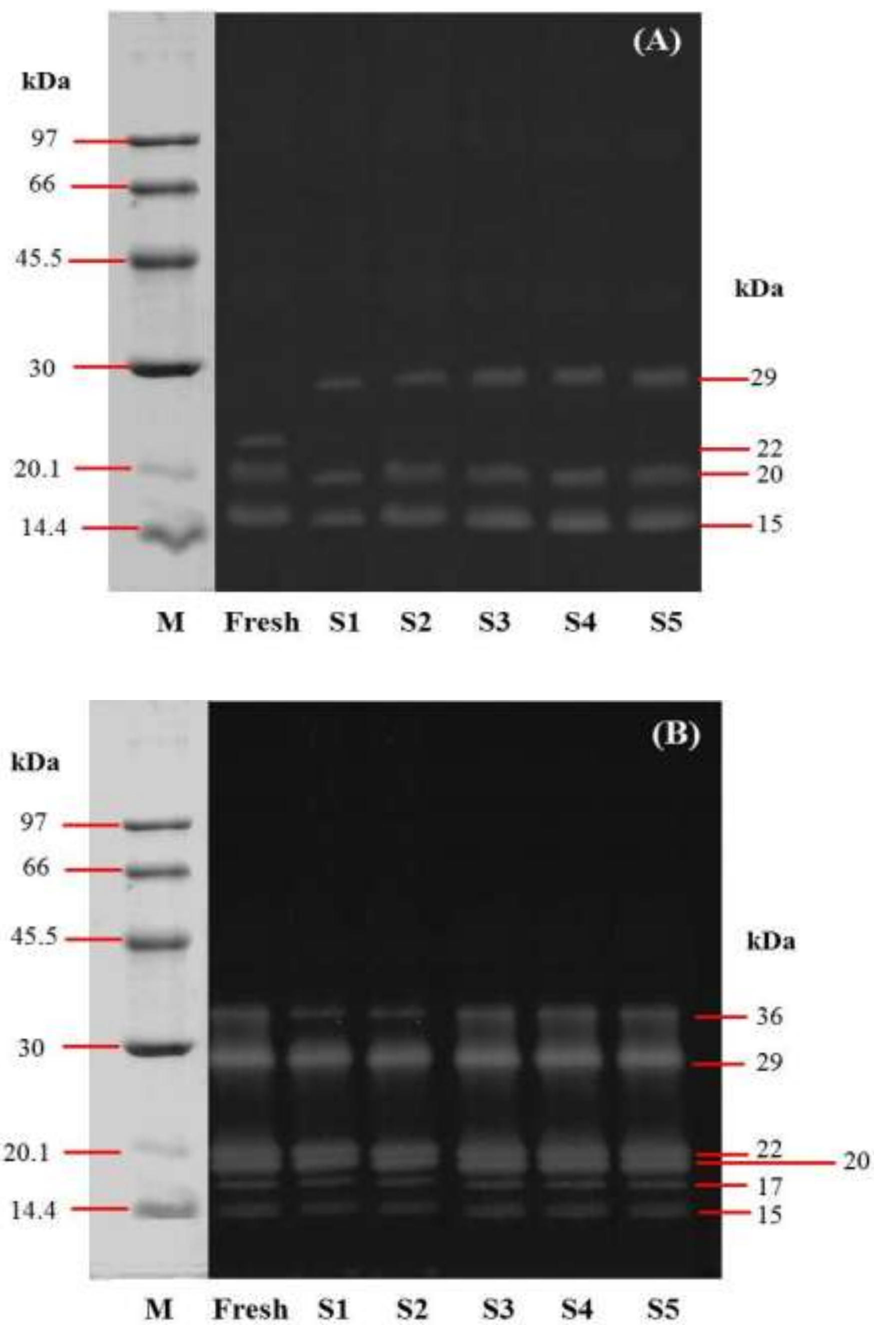

Figure 7. Activity staining of ASF from *A. vulgaris* (A) and *M. lanchesteri* (B) during *Kapi* production. M, molecular weight standard; S1, sample after salting; 2, sample after drying; 3, 4, 5, sample during 10, 20 and 30 days of fermentation, respectively.

4.5.3 Changes in proteins of shrimp during *Kapi* production

4.5.3.1 TCA-soluble peptide and free amino acid contents

The changes in TCA-soluble peptide and free amino acid contents of *A. vulgaris* and *M. lanchesteri* during *Kapi* production are depicted in Figure 8. For fresh shrimp, TCA-soluble peptide and free amino acid contents of *A. vulgaris* and *M. lanchesteri* was 91.01 and 87.57 mmol/g dry weight sample, respectively, suggesting the presence of small peptides or free amino acids in raw material. Pongsetkul *et al.* (2015) reported that fresh *A. vulgaris* had TCA soluble peptide and free amino acid contents of 87.96 mmol/g sample associated with rapid degradation after death, particularly during transportation. Autolysis of shrimp affected the characteristics of *Kapi* to some degree, especially odor characteristics (Pongsetkul *et al.,* 2015). During fermentation, the continuous increase in TCA-soluble peptide and free amino acid contents of both samples was observed up to 30 days ($p < 0.05$). Nevertheless, no differences in TCA-soluble peptide and free amino acid contents in each step of process were noticeable between *A. vulgaris* and *M. lanchesteri* ($p > 0.05$). The results suggested that protein degradation caused by endogenous enzymes as well microbial spoilage was enhanced during *Kapi* production and fermentation. With increasing fermentation time, short chain peptides were produced, thus affecting the characteristics, bioactivities as well as acceptability of *Kapi.*

4.5.3.2 Protein pattern

Changes in protein patterns of *Kapi* produced from *A. vulgaris* and *M. lanchesteri* during processing and fermentation are shown in Figure 9. Fresh shrimp (both *A. vulgaris* and *M. lanchesteri*) contained myosin heavy chain (MHC) as the most dominant protein. In general, shrimp had MHC and actin as the major proteins (Sriket *et al.,* 2012). The band intensity of MHC (MWof 205 kDa) totally disappeared after shrimp were subjected to salting overnight. However, actin band (MWof 45 kDa) still remained. Among all proteins, MHC was most susceptible to proteolytic degradation than other muscle proteins such as actin, troponin and tropomyosin (Benjakul *et al.,* 1997). Actin band of *A. vulgaris* was hydrolyzed continuously, especially during

fermentation. It was noted that actin band of *M. lanchesteri* was drastically degraded and disappeared after drying step. Additionally, proteins or peptides were further hydrolyzed into small peptides. Coincidentally, peptides with MW lower than 30 kDa were slightly increased when the fermentation time increased. The result suggested that different proteins in both species were susceptible to degradation mediated by endogenous proteases and microbial proteases during processing of *Kapi* differently. Cephalothorax of shrimp containing hepatopancreas has been known to be the major source of proteases, mainly serine protease and metalloprotease (Sriket *et al.,* 2012). Sun *et al.* (2014) also noticed that small shrimp or krill contained endogenous proteinase, which can hydrolyze myofibrillar and sarcoplasmic proteins. Overall, proteins of both *A. vulgaris* and *M. lanchesteri* underwent degradation into smaller peptides continuously during processing and fermentation. Those changes might contribute to the characteristics of *Kapi.*

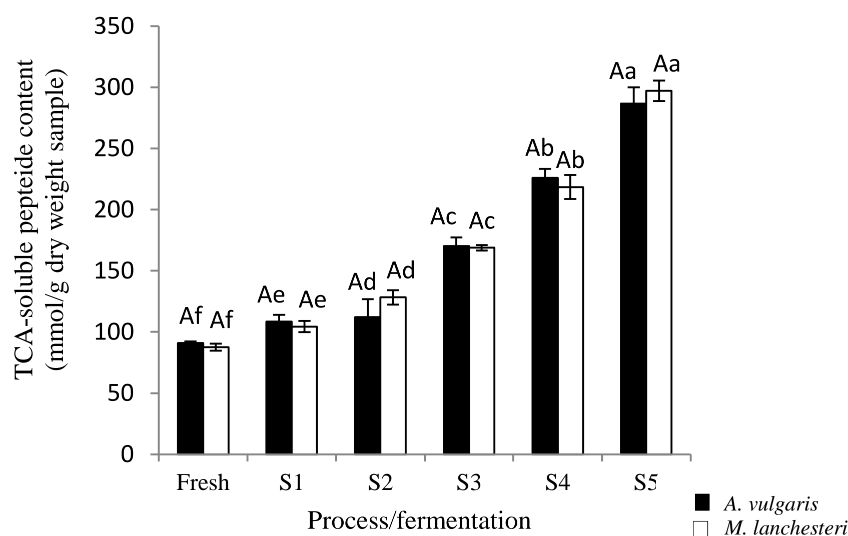

Figure 8. Oligopeptide contents of *A. vulgaris* and *M. lanchesteri* during *Kapi* production. S1, sample after salting; 2, sample after drying; 3, 4, 5, sample during 10, 20 and 30 days of fermentation, respectively.

Bars represent the standard deviation from triplicate determinations. Different uppercase letters on the bars within the same step of production indicate the significant differences ($p < 0.05$). Different lowercase letters on the bars within the same species indicate the significant differences ($p < 0.05$).

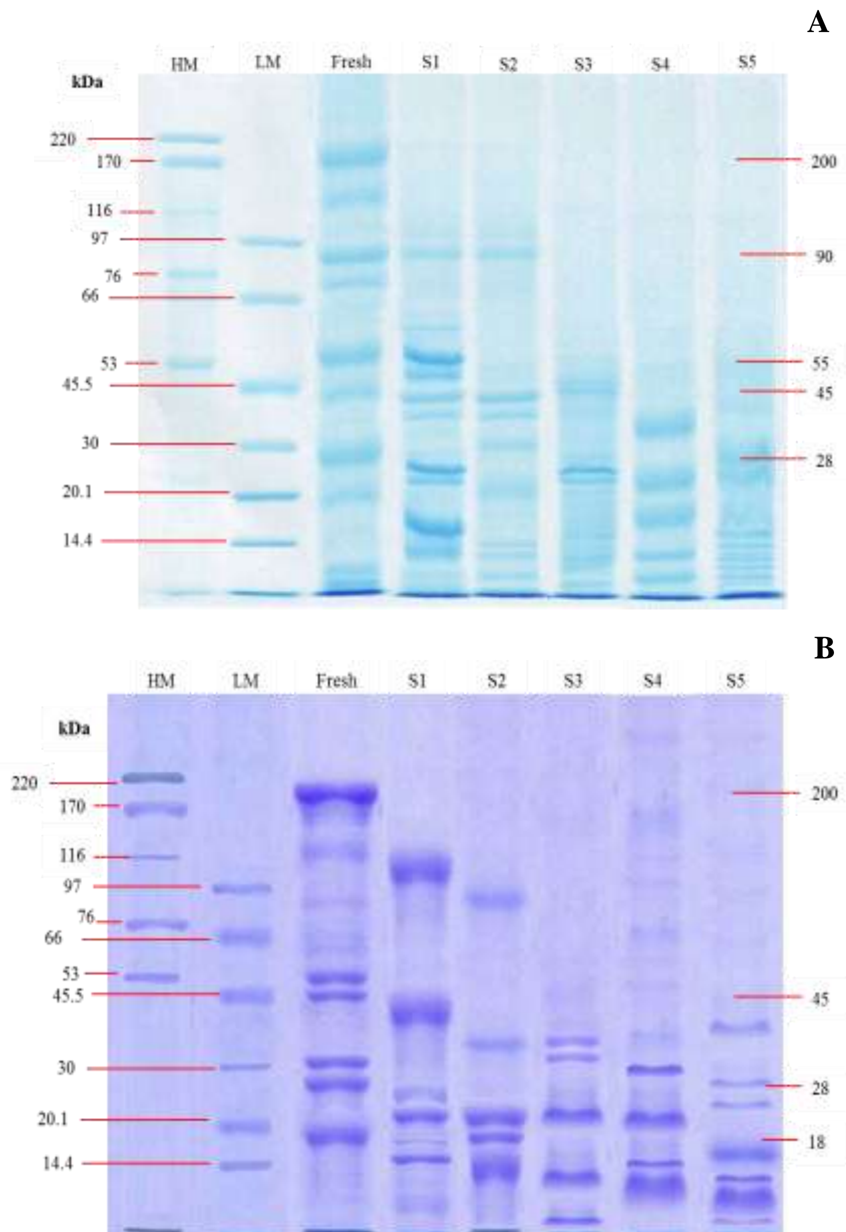

Figure 9. Protein patterns of *A. vulgaris* and *M. lanchesteri* during *Kapi* production. S1, sample after salting; 2, sample after drying; 3, 4, 5, sample during 10, 20 and 30 days of fermentation, respectively. HM, high molecular weight marker, LM, low molecular weight marker.

4.6 Conclusion

Based on the inhibitor study and specific substrates, major proteases from *A. vulgaris* and *M. lanchesteri* were serine proteases (trypsin-like and chymotrypsin-like enzymes) with optimum pH and temperature of 7, 60°C and 8, 60°C, respectively. The molecular weight of these enzymes was in range of 15-36 kDa. Activity of crude extract was decreased with increasing NaCl concentration. In general, proteases still remained during *Kapi* processing/fermentation and played a role in hydrolysis of proteins. *Macrobrachium lanchesteri* had the higher proteolytic activity with larger variety of proteases. Thus, endogenous proteases were involved in protein degradation and had the impact on characteristics of resulting *Kapi.* Additionally, impact of proteases on *Kapi* production depended on shrimp species.

4.7 References

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CHAPTER 5

PROPERTIES OF SALTED SHRIP PASTE (*KAPI*) FROM *ACETES VULGARIS* AS AFFECTED BY POSTMORTEM STORAGE PRIOR TO SALTING

5.1 Abstract

The impact of postmortem storage time of shrimp (*Acetes vulgaris*) on the quality of shrimp and the resulting *Kapi*, or salted shrimp paste, was investigated. When shrimp were stored at room temperature (28-30°C) for up to 15 h, total volatile base, trimethylamine contents, thiobarbituric acid reactive substances, trichloroacetic acid-soluble peptide content and total viable count increased with increasing storage time. Myosin heavy chain and actin underwent degradation throughout the storage. *Kapi* with purplish red color became darker and had higher antioxidative activity when shrimp used as raw material were stored for a longer time prior to salting. *Kapi* showed varying volatile compounds, dependent on the storage time of shrimp and more likely contributed to different flavors. However, *Kapi* showed the lower odor-likeness score when the unfresh shrimp were used. Therefore, storage and handling time of shrimp before salting had the marked influence on the quality of *Kapi.*

5.2 Introduction

Kapi, a typical traditional salted shrimp paste, has been widely consumed in Thailand as a condiment. *Kapi* is normally produced by fermenting small shrimp or krill with solar salt at a ratio of 5:1. The mixture is sun-dried and thoroughly ground before being compacted in a container, usually an earthen jar. Fermentation of salted shrimp paste generally takes place at least 1 month or longer, until the typical aroma is developed (Pongsetkul *et al.,* 2014). Traditionally, krill (*Mesopodopsis orientalis*) is used as a raw material for *Kapi* production. However, krill stocks have dropped by 3% per year since 1990 (Meland and Willassen, 2007). Therefore, an alternative source for production of this product has been searched. Small shrimp belonging to genus *Acetes* have been recently used as raw material to produce shrimp

paste, sauce and other fermented products in Southeast Asian countries. Amongst *Acetes, A. indicus, A. erythraeus, A. vulgaris* and *A. japonicus* have been used widely for making *Kapi* (Hajeb and Jinap, 2015). Generally, shrimp species used for *Kapi* production is determined by availability in each country. For *Kapi* production, some ingredients used can be varied, leading to different characteristics, especially flavor and taste. Different raw materials can bring about products with different appearance, flavor, taste as well as other sensorial properties (Phithakpol and Kasetsat, 1995). The color of *Kapi* varies from a pinkish or purplish grey to dark grayish brown. The consistency also varies, from soft and pasty to dry and hard. *Kapi* is generally preserved for several months (Faithong and Benjakul, 2012).

The delays in salting of shrimp or krill always occur due to the transportation before off-loading or processing. During postmortem storage of shrimp or krill, significant deterioration and loss of nutritional value take place, leading to the distorted quality of the final product (Faithong *et al.,* 2010). The rate of alteration depends on several factors such as the nature of the shrimp species, size, lipid content, state at the moment of capture, microbial load and storage temperature (Sriket *et al.,* 2012). During microbial spoilage, amines, sulfides, alcohols, aldehydes, ketone and organic acids with unpleasant and unacceptable off-flavors are formed (Gram and Dalgaard, 2002). Protein breakdown products such as ammonia, indole, methanethiol, putrescine, trimethylamine and off-odor compounds are also produced by spoilage bacteria (Lakshmanan *et al.,* 2002). Free amino acids and other soluble non-nitrogenous substances can also serve as easily digestible nutrients for microorganisms (Latorre-Moratalla *et al.,* 2011). To prevent the spoilage and formation of undesirable compounds in fishery products, putrefactive microorganisms are inhibited by salt at concentrations above 6-8% (Phithakpol and Kasetsat, 1995). Therefore, the delay in salting might contribute to the change in quality of *Kapi.* Nevertheless, no information regarding the characteristics and quality of *Kapi* from shrimp (*A. vulgaris*) as influenced by postmortem storage time has been reported.

5.3 Objective

To investigate the effect of different postmortem storage times of shrimp (*A. vulgaris*) prior to salting on the characteristics and properties of resulting *Kapi*.

5.4 Materials and methods

5.4.1 Chemicals

All chemicals were of analytical grade. Potassium persulphate, acrylamide, *N,N,N',N'*-tetramethylethylenediamine and bis-acrylamide were procured from Fluka Chemical Co. (Buchs, Switzerland). Malonaldehyde bis (dimethyl acetal), thiobarbituric acid, 2,20-azinobis-(3-ethylbenzothiazoline-6-sulphonic acid) (ABTS), 1,1-diphenyl-2-picrylhydrazyl (DPPH), 2,4,6-tripyridyltriazine and 6-hydroxy-2,5,7,8-tetra-methylchroman-2-carboxylic acid (Trolox) were purchased from Sigma Chemical Co. (St. Louis, MO). Trichloroacetic acid (TCA), sodium chloride, tris (hydroxy-methyl) aminomethane and standard plate count agar were obtained from Merck (Darmstadt, Germany).

5.4.2 Sample collection

Shrimp *A. vulgaris* (average body length 15.6±1.4 mm, average wet weight 0.0413±0.0098 g, n=20) were purchased from a village in Songkhla province, Thailand. Shrimp were transported in ice with a shrimp/ice ratio of 1:2 (w/w) in a polystyrene container to the Department of Food Technology, Prince of Songkla University, Hat Yai, Thailand, within approximately 1 h. Shrimp had 79.97% moisture, 16.52% protein, 0.92% lipid, 1.82% ash and 0.76% carbohydrate as determined by AOAC method (AOAC, 2000).

5.4.3 Quality changes of shrimp during postmortem storage

Upon arrival, shrimp were placed in the basket and stored at room temperature (28-30°C). The sample was periodically taken at 0, 3, 6, 9, 12 and 15 h. The samples were collected and subjected to analyses. Prior to analysis, the whole shrimp were ground to uniformity using a blender (National, Tokyo, Japan).

5.4.3.1 pH determination

The pH of shrimp was measured according to the method of Nirmal and Benjakul (2009) using a pH-meter (Sartorius, Gottingen, Germany).

5.4.3.2 Determination of TVB and TMA contents

Total volatile base (TVB) and trimethylamine (TMA) contents were determined using the Conway micro-diffusion method following the method of Conway and Byrne (1936). The amounts of TVB and TMA were calculated and expressed as mg N/100 g sample.

5.4.3.3 Determination of thiobarbituric acid reactive substances

Thiobarbituric acid reactive substances (TBARS) were determined as described by Nirmal and Benjakul (2009). A standard curve was prepared using malonaldehyde bis (dimethyl acetal) at concentrations ranging from 0 to 2 ppm. TBARS value was calculated and expressed as mg malonaldehyde (MDA)/kg sample.

5.4.3.4 Determination of TCA-soluble peptide content

TCA-soluble peptide content was determined according to the method of Sriket *et al.* (2012) and expressed as μmol tyrosine equivalent/g sample.

5.4.3.5 Protein patterns

Protein patterns were determined by sodium dodecyl sulfate polyacrylamide gel electrophoresis using 4% stacking gel and 10% running gel according to the method of Laemmli (1970). High and low molecular weight protein markers (GE Healthcare U.K. Limited, Buckinghamshire, U.K.) were used to estimate the molecular weight of proteins.

5.4.3.6 Microbiological analysis

Total viable count (TVC) was determined according to the method of BAM (2001). Shrimp samples (25 g) were weighed into a stomacher bag containing

225 ml of peptone water. Blending was performed in a Stomacher 400 Lab Blender (Seward Ltd.,Worthing, U.K.) at high speed for 3 min. Peptone water was used for diluting the samples. Thereafter, the sample diluted in serial 10-fold steps was used for analysis by the spread plate technique on plate count agar (Merck). The plates were incubated at 35°C for 48 h and TVC was recorded.

5.4.4 Effect of postmortem storage time on characteristics and properties of *Kapi*

5.4.4.1 Preparation of *Kapi*

Shrimp (*A. vulgaris*) with different postmortem storage times (0, 6 and 15 h) were used as raw material. Shrimp were mixed with salt at a ratio of 5:1 (w/w) and transferred into the basket and covered with cheesecloth. Then, salted shrimp were allowed to stand at room temperature overnight. The drained samples were mashed or pounded thoroughly and then spread out on plastic or fiberglass mats to dry with sunlight. It was gathered and re-stored in an earthen jar at night and laid out again under the sun. This step was taken for 2-3 days until the samples were disintegrated and turned from pink to dark purplish brown, in which moisture content was in range of 35-40%. Salted shrimp were transferred into earthen jars and covered with plastic bag tightly (close system). Then, samples were allowed to ferment for 1 month (30 days) at room temperature (28-30°C). After 30 days, the obtained *Kapi* was collected for analyses.

5.4.4.2 Proximate composition

Moisture, ash, fat, protein and carbohydrate contents of samples were determined as per AOAC (2000) with the analytical No. of 35.1.13, 35.1.14, 35.1.25, 35.1.15 and 35.1.16, respectively.

5.4.4.3 Salt content

Salt content was determined according to AOAC (2000). The salt content was then calculated and expressed as %NaCl.

5.4.4.4 pH determination

pH of samples was determined as previously described.

5.4.4.5 Color

Color of samples was determined using a colorimeter (ColourFlex, Hunter Lab Reston, VA) and reported in the CIE system. L^* (lightness), a^* (redness/greenness) and b^* (yellowness/blueness) were recorded. Additionally, ΔE^* (total difference of color) and ΔC^* (the difference in chroma) were calculated as described by Pongsetkul *et al.* (2014).

5.4.4.6 Antioxidative activities

- Preparation of water extracts

The extract from *Kapi* samples was prepared according to the method of Peralta *et al.* (2008) with a slight modification. *Kapi* samples (2 g) were mixed with 50 ml of distilled water. The mixtures were homogenized at a speed of 11,000 rpm for 2 min. The homogenates were then subjected to centrifugation at 10,000 rpm for 15 min at room temperature (Model RC-B Plus centrifuge Newtown, CT). The supernatant was collected and adjusted to 50 ml using distilled water. The prepared extracts were assayed for antioxidative activities.

- DPPH radical scavenging activity

DPPH radical scavenging activity was determined according to the method of Faithong and Benjakul (2012) with a slight modification. The standard curve was prepared using Trolox in the range of 10-60 μM. The activity was expressed as μmol Trolox equivalents (TE)/g sample.

- **ABTS radical scavenging activity**

ABTS radical scavenging activity was determined as described by Sun and Tanumihardjo (2007) with a slight modification. A Trolox standard curve (50-600

μM) was prepared and ABTS radical scavenging activity was expressed as μmol TE/g sample.

- Ferric reducing antioxidant power

Ferric reducing antioxidant power (FRAP) was evaluated by the method of Benzie and Strain (1996). The standard curve was prepared using Trolox ranging from 50 to 600 μM. The activity was expressed as μmol TE/g sample.

5.4.4.7 Volatile compounds

Volatile compounds of *Kapi* samples were determined using a solid-phase microextraction gas chromatography mass spectrometry (SPME GC-MS) following the method of Takeungwongtrakul *et al.* (2012). The identified volatile compounds were expressed in the terms of relative abundance.

5.4.4.8 Sensory property

Kapi samples were evaluated by 50 untrained panelists, who consumed *Kapi* regularly. The samples were wrapped with aluminum foil and heated in hot air oven at 60°C for 30 min. Samples were cut to obtain a thickness of 1 cm (2×2 cm^2), then served in white paper plate at room temperature. All samples were coded with three digit random numbers and randomly served. Panelists were instructed to rinse their mouths with water, cucumber or cracker between different samples. Evaluations were made in individual sensory evaluation booths under fluorescent white light. The panelists were asked to assess samples for appearance-liking, color-liking, odor-liking, flavor-liking, texture-liking and overall-liking using a 9-point hedonic scale (1 = dislike extremely, 9 = like extremely) (Mellgard *et al.,* 2007).

5.4.5 Statistical analysis

All experiments were conducted in triplicate. Statistical analysis was performed using one-way analysis of variance. Mean comparison was carried out using Duncan's multiple range test (Steel *et al.,* 1980). SPSS statistic program (SPSS 10.0 for windows, SPSS Inc., Chicago, IL) was used for data analysis.

5.5 Results and discussion

5.5.1 Quality changes of shrimp during postmortem storage

5.5.1.1 Changes in pH

pH of shrimp (*A. vulgaris*) slightly increased from 7.37 to 7.45 with increasing postmortem storage time up to 15 h at room temperature (data not shown). The slightly basic pH might be caused by the degradation products generated during postmortem storage or the formation of volatile base compounds such as ammonia mediated by spoilage microorganisms (Pongsetkul *et al.,* 2014). When the hydrolysis proceeded, the degraded proteins or peptides might undergo decomposition, e.g., via deamination with ease. This could favor the formation of low molecular weight basic compounds. Sriket *et al.* (2012) found that the pH value of freshwater prawn (*Macrobrachium rosenbergii*) was increased during iced storage of 10 days. pH value of shrimp (*Penaeus monodon*) varied from 6.63 to 7.95 during 10 days of iced storage (Rahaman *et al.,* 2001). Changes in pH might be different, depending on a number of factors such as buffering capacity of meat and the liberation of inorganic phosphate and ammonia during enzymatic degradation of adenosine triphosphate (Rahaman *et al.,* 2001).

5.5.1.2 Changes in TVB and TMA contents

The changes in TVB and TMA contents of shrimp during 15 h of postmortem storage at room temperature are presented in Figure 10A and 10B, respectively. TVB content of fresh shrimp (0 h) was 10.15 mg N/100 g sample. As the postmortem time increased, a continuous increase in TVB content was clearly observed up to the end of storage (15 h) ($p < 0.05$). After 15 h, the highest TVB content (74.31 mg N/100 g sample) was obtained. TVB included TMA, dimethylamine, monomethylamine and ammonia (Seibel and Walsh, 2002). An increase in TMA content of shrimp was also noticeable as the postmortem time increased ($p < 0.05$) (Figure 10B). Shrimp stored for 15 h at room temperature showed the highest TMA content (5.89 mg N/100 g sample). TMA is produced by the decomposition of trimethylamine N-oxide (TMAO) due to bacterial spoilage and enzymatic activity

(Hebard *et al.*, 1982). Spoilage bacteria were capable of reducing TMAO to TMA and decarboxylating amino acid to biogenic amines (Dissaraphong *et al.,* 2006). The increases in both TVB and TMA contents were in accordance with the increases in pH (data not shown). The formation of TVB and TMA is generally associated with the growth of specific spoilage bacteria such as *Shewanella putrefaciens*, *Photobacterium phosphoreum* and *Vibrionaceae* (Gram and Dalgaard, 2002). The result suggested that the spoilage caused by bacteria occurred to a higher extent in shrimp, particularly when the storage time increased.

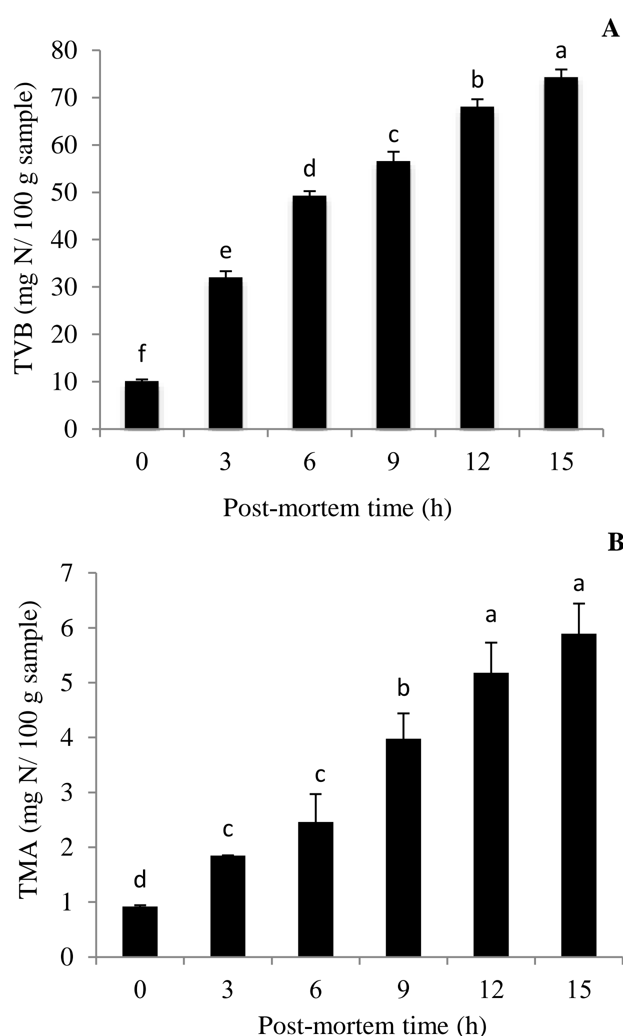

Figure 10. Total volatile base (TVB) (A) and trimethylamine (TMA) contents (B) of shrimp *A. vulgaris* during postmortem storage at room temperature.

Bars represent the standard deviation from triplicate determinations. Different lowercase letters on the bars indicate the significant difference ($p < 0.05$).

5.5.1.3 Changes in TBARS

TBARS values of shrimp during postmortem storage of 15 h are shown in Figure 11A. TBARS value of shrimp increased as postmortem time increased ($p < 0.05$). After 15 h of storage, the highest TBARS value (1.29 mg MDA/kg sample) was found. The result suggested that lipid oxidation took place in shrimp during storage at room temperature. During postmortem storage, autolysis or endogenous enzymatic activity might cause the disruption of the organelles associated with the release of pro-oxidants as well as reactants (Dissaraphong *et al.,* 2006). This led to the enhanced lipid oxidation in the sample. When lipid oxidation occurs, unstable hydroperoxide is formed and decomposed readily to shorter chain hydrocarbon such as aldehydes, which could be detected as TBARS (Benjakul *et al.,* 2005). Lipid oxidation is one of the deteriorative reactions causing the unacceptability of fish and shrimp product. This can be initiated by autoxidation and enzymatic reaction involving lipoxygenase, peroxidase and microbial enzymes (Nirmal and Benjakul, 2009). Additionally, lipid oxidation products more likely contributed to odor and taste of fish and shellfish.

5.5.1.4 Changes in TCA-soluble peptide

The changes in TCA-soluble peptides of shrimp during 15 h of postmortem storage are depicted in Figure 11B. A marked increase in TCA-soluble peptide content was observed within the first 9 h ($p < 0.05$). Nevertheless, no increases in TCA-soluble peptide content were noticeable during 9 and 15 h of storage ($p > 0.05$). The results suggested that protein degradation caused by digestive enzymes and microbial spoilage was enhanced during the first 9 h of storage. For fresh shrimp (0 h), the TCA-soluble peptide content of 87.96 mmol/g sample was found, suggesting the presence of small peptides or free amino acids in *A. vulgaris* or the rapid degradation after death, particularly during transportation. Sriket *et al.* (2012) reported that TCA-soluble peptide content of freshwater prawn (*M. rosenbergii*) was increased during 10 days of iced storage.

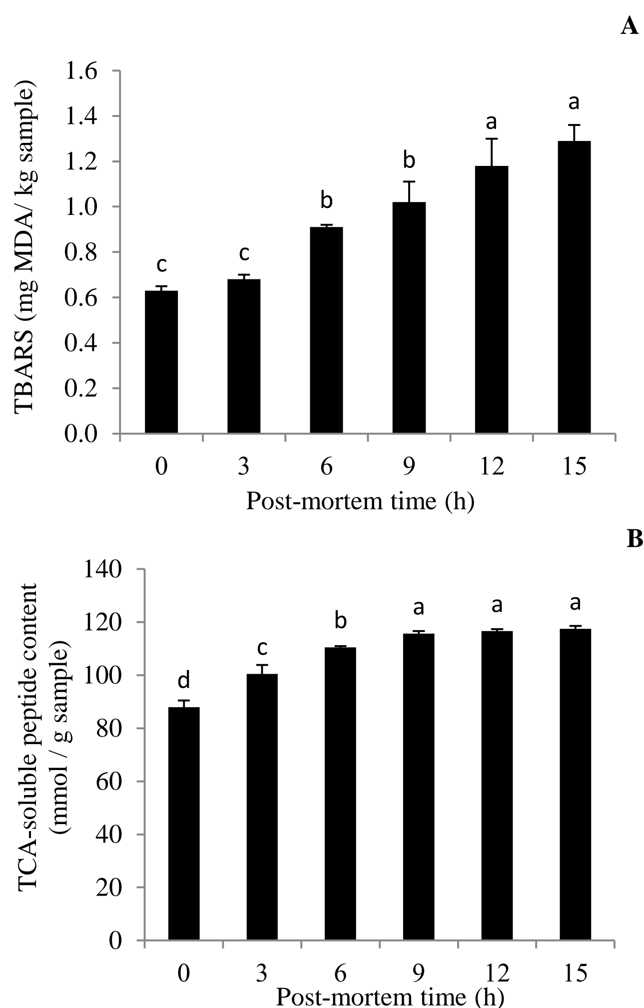

Figure 11. Thiobarbituric acid reactive substances (TBARS) value (A) and TCA-soluble peptide contents (B) of shrimp *A. vulgaris* during postmortem storage at room temperature.

Bars represent the standard deviation from triplicate determinations. Different lowercase letters on the bars indicate the significant difference ($p < 0.05$).

5.5.1.5 Change in total viable count

TVC of fresh shrimp was 2.32 log colony forming units (CFU)/g sample (Figure 12). As the postmortem time increased, a continuous increase in TVC was observed ($p < 0.05$). After 15 h, TVC of shrimp was 4.76 CFU/g sample. In general, TVC increased markedly when the sample was stored at room temperature, in which mesophiles could grow rapidly. Dissaraphong *et al.* (2006) reported that TVC for tuna viscera was 2.93 log cfu/g and reached 5.46 log cfu/g and 5.73 log cfu/g after 8 h of storage in ice and at room temperature, respectively. Those microorganisms were more

likely involved in degradation and decomposition of proteins. Furthermore, those microorganisms might contribute to the flavor of the resulting *Kapi.*

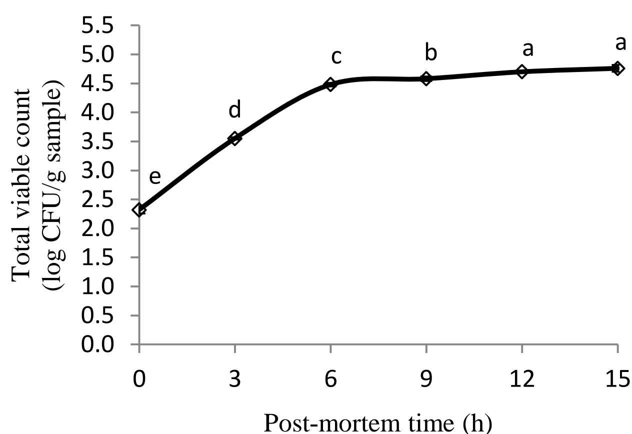

Figure 12. Total viable count (TVC) of shrimp *Acetes vulgaris* during postmortem storage at room temperature.

Different lowercase letters indicate the significant difference ($p < 0.05$)

5.5.1.6 Protein patterns

Protein patterns of whole shrimp with different postmortem storage time are shown in Figure 13. Shrimp had myosin heavy chain (MHC) and actin as the dominant proteins. MHC is generally the major protein in myofibrillar protein and had the molecular weight about 205 kDa (Benjakul *et al.,* 1997). MHC was hydrolyzed continuously throughout the postmortem storage and disappeared when storage time was longer than 6 h. This suggested that MHC underwent degradation drastically through proteolysis after death. It has been reported that MHC was susceptible to proteolytic degradation than other muscle proteins such as actin, troponin and tropomyosin (Benjakul *et al.,* 1997). Sun *et al.* (2014) noticed that small shrimp or krill contained endogenous proteinase which can hydrolyze myofibrillar and sarcoplasmic protein quickly and eventually resulted in low molecular weight peptides. In addition, shrimp cephalothorax containing hepatopancreas has been known to be the major source of proteases. Shrimp were found to contain serine protease and metalloprotease (Garcia-Carreno *et al.,* 1994). For actin, it was found with molecular weight (MW) of 45 kDa. Slight decrease in band intensity of actin was observed within the first 9 h of

storage. However, no further degradation was found during 12-15 h. With an extended storage, proteins or peptides with MW of 70 and 30 kDa were further degraded. Coincidentally, peptide with MW lower than 30 kDa was slightly increased when the storage time increased. Overall, proteins underwent degradation into smaller peptides continuously as the storage time increased.

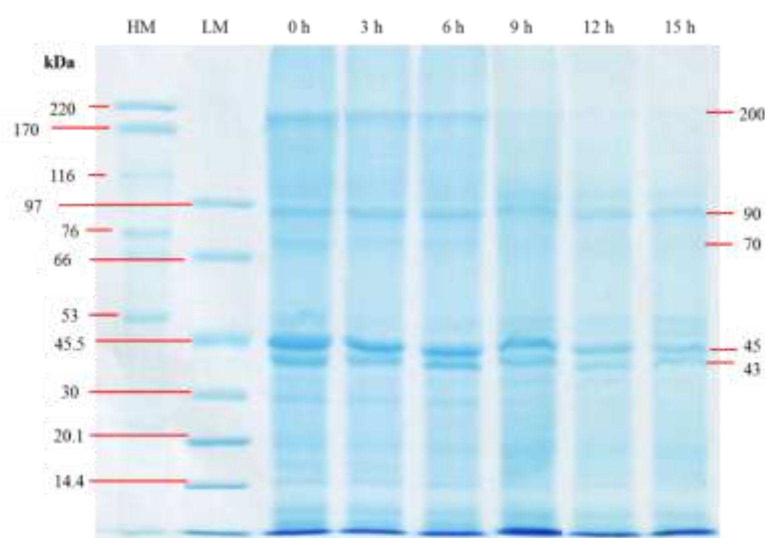

Figure 13. Protein patterns of shrimp *Acetes vulgaris* during post-mortem storage at room temperature. HM: high molecular weight marker, LM: low molecular weight marker.

5.5.2 Effect of postmortem storage time on characteristics and properties of *Kapi*

5.5.2.1 Proximate compositions, salt content and pH

Proximate compositions of *Kapi* produced from shrimp with different postmortem storage times are shown in Table 16. *Kapi* produced from fresh shrimp (0 h) had the lowest moisture content (35.26%) ($p < 0.05$). *Kapi* had slightly higher moisture content when shrimp stored for 6 and 15 h were used as raw material. Based on dry matter, ash was found to be the most dominant constituent (50.76-53.38%), followed by protein (40.79-44.43%). Fat content in *Kapi* ranged from 2.86 to 3.06%. Salted shrimp paste oil was reported to be rich in polyunsaturated fatty acid (Montano

et al., 2006), indicating its nutritive value. *Kapi* was also rich in astaxanthin (Pongsetkul *et al.,* 2014). There were no differences in fat, ash and salt content ($p > 0.05$) among *Kapi* samples produced from shrimp with different postmortem storage times. Nevertheless, *Kapi* produced from fresh shrimp (0 h) had the lower protein content but higher carbohydrate content than those prepared from shrimp stored for 6 and 15 h ($p < 0.05$). Carbohydrate content of all samples was in ranges of 0.74-5.59% (dry weight basis). Normally, carbohydrate content of shrimp was varied, depending on species and maturation stage. Carbohydrate content of Jinga shrimp (*Metapenaeus affinis*) was 0.96% (dry weight basis) (Dincer and Aydin, 2014), while the fresh water prawn (*Macrobrachium rosenbergii*) and Indian white shrimp (*Penaeus indicus*) had the carbohydrate content of 5.50 and 2.64% (dry weight basis), respectively (Reddey and Reddey, 2014; Ravichandra *et al.,* 2009). Low carbohydrate content found in *Kapi* samples indicated that no carbohydrate ingredients were used for *Kapi* production. Pongsetkul *et al.* (2014) found the wide range of carbohydrate content in commercial *Kapi* (4.90-32.48% dry weight basis). It was noted that *Kapi* produced from shrimp at time 0 had the higher carbohydrate content than others. This was plausibly due to the accumulation of glycogen at high level in fresh shrimp. During storage at room temperature, the glycogen could undergo glycolysis as indicated by the decreased carbohydrate content. High salt used for *Kapi* production contributed to high ash content (50.76-53.38%) in all samples. It was noted that ash content was higher than salt content in all samples. Since whole shrimp containing shells were used, $CaCO_3$ or other minerals in shell contributed to the inorganic matter, in addition to NaCl added as the preservative.

Kapi produced from shrimp with longer postmortem storage time showed the higher pH ($p < 0.05$). *Kapi* produced from fresh shrimp (0 h) had the lowest pH value (7.10) and the highest pH value (7.81) was obtained in *Kapi* produced from shrimp stored for 15 h prior to salting. pH of *Kapi* was in accordance with those of shrimp used as raw material, in which pH was also increased with increasing postmortem storage time. Degradation products, particularly volatile base compounds generated during postmortem storage, most likely contributed to the increases in pH of resulting *Kapi*.

Table 16. Proximate composition, pH, color and antioxidative properties of *Kapi* produced from shrimp *A. vulgaris* with different post-mortem times

Compositions/Properties	Post-mortem time		
	0 h	6 h	15 h
Moisture	35.26±1.17^{b}	37.45±1.31^{a}	38.39±0.60^{a}
Ash	32.86±0.89^{a}	33.39±0.87^{a}	31.93±1.27^{a}
	(50.76±1.38)^{A}	(53.38±1.40)^{A}	(51.83±2.06)^{A}
Protein	26.41±0.56^{a}	26.73±1.91^{a}	27.37±0.69^{a}
	(40.79±0.86)^{B}	(42.74±0.86)^{A}	(44.43±1.12)^{A}
Fat	1.85±0.11^{a}	1.91±0.09^{a}	1.85±0.07^{a}
	(2.86±0.17)^{A}	(3.06±0.14)^{A}	(3.00±0.12)^{A}
Carbohydrate	3.62±0.21^{a}	0.52±0.19^{b}	0.46±0.22^{b}
	(5.59±0.32)^{A}	(1.82±0.44)^{B}	(0.74±0.19)^{B}
Salt content	22.47±1.02^{a}	22.42±0.72^{a}	22.34±0.27^{a}
	(34.71±1.58)^{A}	(35.84±1.16)^{A}	(36.26±0.45)^{A}
pH	7.10±0.01^{c}	7.34±0.01^{b}	7.81±0.15^{a}
L^*	57.92±0.44^{a}	51.56±0.91^{b}	49.31±0.26^{c}
a^*	5.16±0.10^{c}	6.16±0.11^{b}	7.20±0.09^{a}
b^*	15.73±0.55^{a}	10.90±0.43^{b}	8.87±0.18^{c}
ΔE^*	39.26±0.23^{c}	43.85±0.96^{b}	45.76±0.51^{a}
ΔC^*	15.63±0.11^{a}	11.60±0.23^{b}	10.51±0.19^{c}
DPPH radical scavenging activity**	1.40±0.22^{c}	2.58±0.28^{b}	3.28±0.30^{a}
ABTS radical scavenging activity**	8.32±0.13^{c}	10.21±0.33^{b}	11.58±0.25^{a}
FRAP**	15.89±0.04^{a}	15.91±0.38^{a}	16.16±0.73^{a}

** (μmol TE/g sample)
Values in parentheses indicate the content expressed, based on dry weight.
Mean ± SD from triplicate determinations. Different lowercase and uppercase superscripts in the same row indicate the significant difference ($p < 0.05$).

5.5.2.2 Color

Kapi produced from shrimp with different postmortem storage times had different colors. L^* (lightness), a^* (redness), b^* (yellowness), ΔE^* (total difference in color) and ΔC^* (difference in chroma) were in the range of 49.31-57.92, 5.16-7.20, 8.87-15.73, 39.26-45.76 and 10.51-15.63, respectively. *Kapi* produced by shrimp with 15 h of postmortem times showed the highest a^* and ΔE^* ($p < 0.05$), however, it had lower b^*, L^* and ΔC^* values ($p < 0.05$). *Kapi* produced by shrimp with 15 h of postmortem time was dark purplish brown. During the delay in salting, biochemical change, especially enzymatic reactions, occurred. Those included browning reaction induced by polyphenoloxidase (PPO). PPO has been known to induce the hydroxylation

of phenols with subsequent polymerization, in which melanin is formed (Nirmal and Benjakul, 2009). Additionally, Maillard reaction might contribute to the darker color of *Kapi.* During extended storage or during fermentation, free amino groups at higher level in sample kept for a long time could serve as reactants for glycation process. As a result, the darker color was found in *Kapi* produced from shrimp stored for 15 h prior to salting. This was also in accordance with the increases in a^* and ΔE^* value. Most of the nitrogenous compounds in *Kapi* are free amino acids and small peptides, which contribute to the brown color development (Lopetcharat *et al.,* 2001). Carbohydrate derivatives, such as glucose-6-phosphate and other substances present in the metabolic pathways, can also act as reactants to initiate the Maillard reaction (Kawashima *et al.,* 1977). Therefore, postmortem time prior to salting played a role in the color of *Kapi* produced from *A. vulgaris.*

5.5.2.3 Antioxidative activities

Antioxidative activities of the water extracts of *Kapi* produced from shrimp with different postmortem times are shown in Table 16. DPPH and ABTS radical scavenging activities of water extracts increased when the postmortem time of shrimp used as raw material of *Kapi* production increased. DPPH radical scavenging has been used to indicate hydrogen-donating ability of the extract (Benjakul *et al.,* 2005). Peptides or free amino acids in *Kapi* possessed the ability to donate the hydrogen atom to free radicals, in which the propagation process of oxidation could be retarded (Faithong *et al.,* 2010). Water extract of *Kapi* produced from shrimp with 15 h of postmortem time showed the highest DPPH radical scavenging activity (3.28 μmol TE/g sample), whereas that of *Kapi* produced from fresh shrimp (0 h) had the lowest DPPH radical scavenging (1.40 μmol TE/g sample) ($p < 0.05$). Thus, postmortem time of shrimp determined hydrogen donating ability of resulting *Kapi*.

ABTS radical scavenging activity of water extracts of all samples showed similar trend with DPPH radical scavenging activity. In general, ABTS radical scavenging assay has been used to determine both hydrophilic and lipophilic antioxidants (Sun and Tanumihardjo, 2007). Water extract of *Kapi* produced from shrimp with 15 h postmortem time showed the highest ABTS radical scavenging activity (11.58 μmol TE/g sample), whereas *Kapi* produced from fresh shrimp (0 h) had

the lowest ABTS radical scavenging activity (8.32 μmol TE/g sample) ($p < 0.05$). The result reconfirmed that the delay in salting of shrimp was associated with the increased radical scavenging activities of *Kapi.* Increasing degradation or breakdown of protein as indicated by increasing TCA-soluble peptide content (Figure 11B) and more degradation (Figure 13) were in accordance with increasing antioxidative capacity. Generally, hydrolysis was progressed during fermentation, leading to the accumulation of hydrolyzed peptides and amino acids. Low molecular weight peptides and amino acids from salted shrimp paste have been reported to possess antioxidant activity (Peralta *et al.,* 2008; Faithong *et al.,* 2010; Faithong and Benjakul, 2012; Pongsetkul *et al.,* 2014). Furthermore, Maillard reaction product (MRP) intermediates and final products in *Kapi* might partially contribute to the antioxidative activity.

No differences in FRAP were observed among all *Kapi* extracts tested ($p > 0.05$). Thus, those peptides showing both DPPH and ABTS radical scavenging activities might not possess the reducing power, whereas peptides with reducing power were present at similar level in all *Kapi* samples. Therefore, antioxidative activity of *Kapi* was governed by postmortem time prior to salting and the activity was also determined by amount and type of peptides generated during storage and fermentation.

5.5.2.4 Volatile compounds

Different volatile compounds of *Kapi* produced from shrimp with different postmortem times were determined using a SPME GC-MS (Table 17). Forty-three volatile compounds were isolated and identified. Those compounds could be classified as aldehydes (4), ketones (8), alcohols (10), nitrogen-containing compounds (8), hydrocarbons (5) and others (8). Flavoring compounds in salted shrimp paste play the most important part in the acceptance and preference by consumers (Van-Ba *et al.,* 2012). The most important mechanisms responsible for these volatile compounds are the degradation of lipid (mainly oxidation), Maillard reaction, interaction between Maillard reaction products with lipid-oxidized products and others (Van-Ba *et al.,* 2012).

Table 17. Volatile compounds of *Kapi* produced from shrimp *A. vulgaris* with different post-mortem times

Volatile compounds (Abundance×10^6)	Post-mortem time		
	0 h	6 h	15 h
Aldehydes			
3-methyl-butanal	67.74	65.91	132.98
Pentanal	39.76	121.43	195.76
4-heptanal	73.98	ND	99.5
Benzaldehyde	72.91	207.16	216.06
Ketones			
1-phenyl-ethanone	93.42	101.5	169.13
1-(2-pyridinyl)-ethanone	154.05	94.49	166.12
1-(2-aminophenyl)-ethanone	125.81	98.19	130.55
2-Heptanone	58.58	95.9	82.58
6-Methyl-5-hepten-2-one	ND	40.98	94.82
2-Octanone	ND	83.9	160.4
6-Octen-2-one	ND	162.23	282.93
3,5-Octadien-2-one	48.6	74.38	164.91
Alcohols			
Benzenemethanol	32.49	95.6	128.36
2-(3-cyclohezen-1-yl)-ethanol	46.46	77.28	97.94
1-Butanol	ND	ND	100.1
3-methyl, 1-butanol	ND	96.58	ND
1-Pentanol	135.81	144.45	110.44
1-Penten-3-ol	124.83	278.06	64.75
1-Hexanol	161.48	131.54	133.75
1-Octanol	51.26	ND	ND
1-Octen-3-ol	269.93	392.54	194.29
Octa-1,5-dien-3-ol	250.85	340.51	122.01
Nitrogen-containing compounds			
Methyl-pyrazine	570.54	ND	ND
Trimethyl-pyrazine	512.78	902.34	751.92
2,5-dimethyl-pyrazine	809.95	804.19	877.92
2,6-dimethyl-pyrazine	437.64	491.63	477.21
2-ethyl-5-methyl-pyrazine	830.76	805.85	203.6
2-ethyl-2,5-dimethyl-pyrazine	ND	ND	146.87
3-ethyl-2,5-dimethyl-pyrazine	860.99	899.81	656.14
2-methyl-5-propyl-pyrazine	250.4	193.67	ND
Hydrocarbon			
2,6,10,14-tetramethyl-pentadecane	ND	228.89	232.72
Bicyclo-octane	ND	176.4	231.58
2,2-dimethyl-4-decane	ND	252.24	ND
2-Undecyne	ND	440.59	449.22
3-Dodecyne	205.56	ND	ND

ND: non-detectable.

Table 17. Volatile compounds of *Kapi* produced from shrimp *A. vulgaris* with different post-mortem times (Cont)

Volatile compounds (Abundance×10^6)	Post-mortem time		
	0 h	6 h	15 h
Others			
3-methyl-butanoic acid	ND	ND	130.7
Ethyl-ester-pentadecanoic acid	107	119.67	169.82
Methyl-ester-heptadecanoic acid	ND	235.46	ND
Octanoic acid	164.13	161.01	168.61
Methyl-ester-octadecanoic acid	155.37	ND	268.76
Isosorbide	136.53	90.56	139.42
Phenol	247.59	233.15	788.96
1H-Indole	180.45	197.22	638.16

ND: non-detectable.

Aldehydes were the compounds found in *Kapi*, regardless of postmortem time prior to salting. The chemicals 3-methyl-butanal, pentanal, 4-heptanal and benzaldehyde were detected in *Kapi*. The presence of these compounds is related with lipid oxidation. Higher abundance in all aldehydes was found in sample produced from shrimp stored for 15 h before salting. During spoilage, chemical deterioration including lipid oxidation took place. Hydroperoxide could be formed from free fatty acids released from triglycerides by putrefactive bacteria (Peralta *et al.,* 1996). In general, aldehydes and ketones were more likely generated from lipid oxidation during fermentation (Takeungwongtrakul *et al.,* 2012). The highest aldehydes in *Kapi* produced from shrimp with 15 h of postmortem time were coincidental with highest TBARS value (Figure 11A) and microbial count (Figire 12). Benzaldehyde was found in all samples. Benzaldehyde was reported to have a pleasant almond, nutty and fruity aroma (Cha and Cadwallader, 1995). Branched short chain aldehydes or aromatic aldehydes plausibly resulted from deamination of amino acids (Casaburi *et al.,* 2008). The sensory characteristics of aldehydes are mainly associated with a fatty aroma. Straightchain aldehydes have been described as giving grass and rancid notes (Casaburi *et al.,* 2008). Fat (1.41-3.67%) in *Kapi* (Pongsetkul *et al.,* 2014) more likely underwent oxidation, leading to the formation of aldehydes.

Ketones were also found in *Kapi*. The chemicals 1-phenyl-ethanone, 1-(2-pyridinyl)-ethanone, 1-(2-aminophenyl)-ethanone, 2-heptanone and 3,5-octadien-2-one were found in all samples and the higher abundance was obtained in *Kapi* produced from shrimp with 15 h of postmortem time. Ketones seem to be responsible for the cheesy note in fish sauce odor (Peralta *et al.,* 1996). However, such compounds with low concentrations and high odor threshold values might not contribute to flavor of this product (Cha and Cadwallader, 1995). It was noted that 6-methyl-5-heptone-2-one, 2-octanone and 6-octen-2-one were found only in *Kapi* produced from shrimp stored for 6 and 15 h, but not detected in sample produced from fresh shrimp (0 h). The result confirmed that postmortem storage was an important factor determining the formation of ketones in *Kapi.*

Normal and branched alcohols detected in *Kapi* included 1-pentanol, 1-penten-3-ol, 1-hexanol, 1-octen-3-ol and octa-1,5-dien-3-ol. These alcohols might be produced from microbial fermentation or the degradation products from lipid oxidation. Alcohols were quite low in abundance in *Kapi*. The abundance of individual alcohol varied with postmortem time of shrimp used for *Kapi* production. In general, alcohols might not have a paramount impact on *Kapi* flavor because of their high flavor thresholds (Cha and Cadwallader, 1995).

Eight nitrogen-containing compounds were found in *Kapi*, which were all pyrazine derivatives. Trimethylpyrazine, 2,5-dimethyl pyrazine, 2,6-dimethyl pyrazine, 2-ethyl-5-methyl pyrazine and 3-ethyl-2,5-dimethyl pyrazine were detected in all samples. Pyrazines were reported to be formed by Maillard reaction through strecker degradations from various nitrogen sources such as amino acids (Jaffres *et al.,* 2011). It was found that pyrazines could be responsible for the burnt and sweet odors of Vietnamese fish sauce (*nouc-mam*) (Lopetcharat *et al.,* 2001). Methylpyrazine was found only in *Kapi* produced with fresh shrimp (0 h), whilst 2-ethyl-2,5-dimethyl-pyrazine was found only in *Kapi* produced from shrimp stored for 15 h. The presence of pyrazine indicated that browning reaction mediated by Maillard reaction occurred in *Kapi* during fermentation. This might contribute to flavor, color as well as antioxidative activity of *Kapi*.

Some hydrocarbon compounds including 2,6,10,14-tetramethyl pentadecane and bicyclo-octane, 2-undecyne were found in *Kapi* produced from shrimp stored for 6 and 15 h prior to salting. Nevertheless, 3-dodecyne was present only in *Kapi* produced from fresh shrimp. Alkanes and alkenes are mainly formed from lipid auto-oxidation of fatty acids released from triglycerides (Latorre-Moratalla *et al.,* 2011). Additionally, all samples contained phenol, which was reported to give an undesirable aroma in seafood (Cha and Cadwallader, 1995). *Kapi* produced from shrimp with 15 h of postmortem times had the higher abundance in phenol than other samples. Furthermore, all samples also consisted of indole. The highest indole was also found in *Kapi* produced from shrimp with 15 h of postmortem time. Indole is the degradation product from tryptophan and has been used as the index for shrimp spoilage (Lakshmanan *et al.,* 2002). The result confirmed that shrimp stored at room temperature for a longer time more likely underwent spoilage to some degrees as indicated by the increased indole in the resulting *Kapi*.

Based on volatile compounds, *Kapi* produced from shrimp with different postmortem times showed varying volatile compounds. This might be associated with different flavors of *Kapi*.

5.5.2.5 Sensory properties

Table 18 shows likeness scores of *Kapi* produced from shrimp with different postmortem times. *Kapi* produced from shrimp with 6 or 15 h of postmortem time had slightly higher appearance-liking score than that prepared from fresh shrimp ($p < 0.05$). The highest color-liking score (7.90) was found in *Kapi* prepared from shrimp with 15 h postmortem time ($p < 0.05$). The result suggested that *Kapi* with browner or darker color was more desirable. *Kapi* from shrimp having 15 h postmortem time had the lowest L^* but highest a^*-value ($p < 0.05$) (Table 16). However, the lowest odor-liking score (6.14) was found in this sample. Odor of *Kapi* was possibly governed by different volatile compounds. *Kapi* produced from shrimp stored for 15 h prior to salting might have undesirable volatiles, which were associated with deterioration products, particularly formed during the extended postmortem storage. Aldehydes and ketones along with indole were generally found at high abundance in *Kapi* prepared from shrimp stored for 15 h before salting. However, *Kapi* produced from shrimp with

15 h of postmortem times had the similar overall-liking score to those produced from fresh shrimp and shrimp stored for 6 h. Overall, sensorial characteristics of *Kapi* samples could be influenced by the freshness of the raw material used.

Table 18. Likeness score of *Kapi* produced from shrimp *A. vulgaris* with different post-mortem times.

Attributes	Post-mortem time		
	0 h	6 h	15 h
Appearance	6.05 ± 1.07^{b}	7.38 ± 0.80^{a}	7.76 ± 1.04^{a}
Color	5.95 ± 1.11^{c}	7.19 ± 0.75^{b}	7.90 ± 0.77^{a}
Odor	7.14 ± 1.11^{a}	7.24 ± 0.77^{a}	6.14 ± 1.77^{b}
Texture	6.33 ± 0.77^{b}	7.33 ± 0.86^{a}	7.52 ± 0.93^{a}
Flavor	6.86 ± 1.06^{a}	7.48 ± 0.93^{a}	7.05 ± 1.12^{a}
Overall	6.71 ± 0.85^{b}	7.38 ± 0.74^{a}	6.95 ± 1.24^{ab}

Values are given as mean ± SD (n = 3). Different lowercase superscripts within the same row indicate the significant differences ($p < 0.05$).

5.6 Conclusion

Shrimp (*A. vulgaris*) could be used as an alternative raw material for *Kapi* production. Postmortem storage at room temperature prior to salting could allow the deterioration to proceed. Protein hydrolysis and decomposition as well as lipid oxidation took place when shrimp were stored before salting. Those alternations affected the color, antioxidative activities, volatile compounds as well as sensory property of resulting *Kapi*. In general, the delay in salting up to 15 h led to the lower odor-likeness of resulting *Kapi*. Furthermore, the different freshness of shrimp was associated with varying characteristics and properties of *Kapi*.

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CHAPTER 6

EFFECT OF POSTMORTEM STORAGE PRIOR TO SALTING ON QUALITY OF SALTED SHRIMP PASTE (*KAPI*) PRODUCED FROM *MACROBRACHIUM LANCHESTERI*

6.1 Abstract

Effect of post-mortem storage time of shrimp (*Macrobrachium lanchesteri*) on quality of shrimp and the resulting *Kapi*, salted shrimp paste, was investigated. Shrimp underwent deterioration when stored at room temperature (28-30°C) up to 18 h as indicated by the increases in pH, total volatile base (TVB), trimethylamine (TMA) contents, thiobarbituric acid reactive substances (TBARS) and total viable count (TVC). Protein degradation was more pronounced as evidenced by the decrease in band intensity of myosin heavy chain with coincidental increase in TCA soluble peptides. Post-mortem storage time of shrimp prior to salting had impact on the quality of resulting *Kapi*. With increasing storage time, *Kapi* became browner with higher antioxidative activity. Also, volatile compounds including aldehydes, ketones, alcohols and pyrazines increased continuously. The highest and lowest overall likeness scores were obtained for *Kapi* prepared from shrimp stored for 6 and 18 h, respectively. Therefore, post-mortem storage time of shrimp used as raw material had the marked influence on quality of resulting *Kapi*.

6.2 Introduction

Kapi, a Thai traditional fermented shrimp paste, is widely consumed as a condiment. In general, small shrimp or krill have been used as the main raw materials to produce *Kapi*. Shrimp or krill tissues undergo enzymatic breakdown during the fermentation and bacterial action assists in proteolysis and flavor development (Hajeb and Jinap, 2015). Peptides in salted shrimp paste possessed bioactive activities, especially antioxidant activities (Peralta *et al.,* 2008; Faithong and Benjakul, 2012; Pongsetkul *et al.,* 2014; Pongsetkul *et al.,* 2015).

Kapi is made by mixing shrimp or krill with salt at a ratio of 3-5:1, followed by sun-drying. Sun-dried salted shrimp is thoroughly ground before being compacted in a container, usually earthen jar and allowed to ferment for at least 1 month or longer until the typical aroma is developed (Pongsetkul *et al.,* 2014). Salting and drying processes increase the shelf-life and flavor intensity of the product. Putrefactive microorganisms are inhibited by salt at concentrations above 6 to 8% (Phithakpol, 1993). The delay in salting might contribute to the differences in quality of resulting *Kapi*.

However, the planktonous shrimp or krill (*Mesopodopsis orientalis*), which is the traditional raw material for *Kapi* production, have dropped by 3% per year since 1990 (Meland and Willassen, 2007). Therefore, the alternative raw material for *Kapi* production has been searched. Small shrimp (*Macrobrachium lanchesteri*) is generally byproducts from commercial fishing and it usually founds in southern part of Thailand throughout the year. This species seem to be a potential alternative source for making *Kapi* because of its availability and low price. Generally, species of shrimp, the quantity of salt used, and the treatment of raw materials prior to fermentation, can be varied, leading to the different characteristics, especially flavor and taste (Phithakpol, 1993). Nevertheless, no information regarding the characteristics and quality of *Kapi* from shrimp (*M. lanchesteri*) as influenced by post-mortem storage before salting has been reported.

6.3 Objective

To investigate the effect of different post-mortem storage time of shrimp *M. lanchesteri* prior to salting on the characteristics and some properties of resulting *Kapi.*

6.4 Materials and methods

6.4.1 Sample collection

Shrimp *M. lanchesteri* (average body length 27.5±1.9 mm, average wet weight 0.0701±0.0107 g, n=20) were purchased from a village in The-Pha, Songkhla, Thailand. Shrimp were transported in ice with a shrimp/ice ratio of 1: 2 (w/w) in a

polystyrene container to the Department of Food Technology, Prince of Songkla University, Hat Yai, Thailand, within approximately 2 h. Whole shrimp had 80.04% moisture, 16.31% protein, 0.58% lipid, 2.26% ash and 0.81% carbohydrate as determined by AOAC method (AOAC, 2000).

6.4.2 Quality changes of shrimp during postmortem storage

Upon arrival, shrimp were placed in the basket and stored at room temperature (28-30°C). Shrimp were periodically taken at 0, 3, 6, 9, 12, 15 and 18 h. The collected samples were pooled and blended using a blender (National, Tokyo, Japan) prior to analysis.

6.4.2.1 pH determination

The pH of shrimp was measured according to the method of Nirmal and Benjakul (2009) using a pH-meter (Sartorius, Gottingen, Germany).

6.4.2.2 Determination of TVB and TMA contents

Total volatile base (TVB) and trimethylamine (TMA) contents were determined using the Conway micro-diffusion method following the method of Conway and Byrne (1936). The amounts of TVB and TMA were calculated and expressed as mg N/100 g sample.

6.4.2.3 Determination of thiobarbituric acid reactive substances

Thiobarbituric acid reactive substances (TBARS) were determined as described by Nirmal and Benjakul (2009). A standard curve was prepared using malonaldehyde bis (dimethyl acetal) at concentrations ranging from 0 to 2 ppm. TBARS value was calculated and expressed as mg malonaldehyde (MDA)/kg sample.

6.4.2.4 Determination of TCA-soluble peptide content

Oligopeptide content of samples was determined according to the method of Sriket *et al.* (2012). Soluble oligopeptide content in the supernatant was

measured according to the Lowry method (Lowry *et al.,* 1951) and expressed as µmol tyrosine equivalent/g sample.

6.4.2.5 Protein patterns

Protein patterns were determined by sodium dodecyl sulfate polyacrylamide gel electrophoresis using 4% stacking gel and 10% running gel according to the method of Laemmli (1970). High and low molecular weight protein markers (GE Healthcare U.K. Limited, Buckinghamshire, U.K.) were used to estimate the molecular weight of proteins.

6.4.2.6 Microbiological analysis

Shrimp (25 g) were transferred into a stomacher bag containing 225 ml of peptone water. Blending was performed in a Stomacher 400 Lab Blender (Seward Ltd., Worthing, UK) at high speed for 3 min. Peptone water was used for diluting the samples. Thereafter, the sample diluted in serial 10-fold steps was used for analysis by the spread plate technique on plate count agar (PCA-Merck). The plates were incubated at 35°C for 48 h. Total viable count (TVC) was recorded and expressed as log CFU/g sample (BAM, 2001).

6.4.3 Effect of postmortem storage time on characteristics and properties of *Kapi*

6.4.3.1 Preparation of *Kapi*

Shrimp with different post-mortem storage times (0, 6, 12 and 18 h) were used as raw material. Shrimp were mixed with salt at a ratio of 5:1 (w/w) and transferred into the basket and covered with cheesecloth. Salted shrimp were made as per the method of Pongsetkul *et al.* (2015). After 30 days, the obtained *Kapi* samples were taken for analyses.

6.4.3.2 pH determination

pH of samples was determined as previously described.

6.4.3.3 Formal, ammonia and amino nitrogen contents

Formal, ammonia and amino nitrogen contents were determined by the titration method as described by Pongsetkul *et al.* (2014). The results were calculated and expressed as mg N/g sample.

6.4.3.4 Color

Color of samples was determined using a colourimeter (ColourFlex, Hunter Lab Reston, VA, USA) and reported in the CIE system. L^* (lightness), a^* (redness/greenness), and b^* (yellowness/blueness) were recorded. Additionally, ΔE^* (total difference of color) and ΔC^* (the difference in chroma) were calculated.

6.4.3.5 Browning index and Maillard reaction product

- Preparation of water extract

Water extract was prepared according to the method of Peralta *et al.* (2008) with a slight modification. *Kapi* (2 g) was mixed with 50 ml of distilled water. The mixtures were homogenised at a speed of 10,000×*g* for 2 min. The homogenates were then subjected to centrifugation at 13,000×*g* for 15 min at room temperature. The supernatant was collected. The pellet was re-extracted in the same manner. The supernatants were combined and adjusted to 50 ml using distilled water.

- Measurement of browning intensity

The browning intensity of the extract was measured as per the method of Pongsetkul *et al.* (2014). Appropriate dilution was made using distilled water and the absorbance was measured at 420 nm using the UV-1601 spectrometer (Shimadzu, Kyoto, Japan).

- Measurement of Maillard reaction products

Fluorescent intermediate products from Maillard reaction in the extract were determined as described by Pongsetkul *et al.* (2014). A_{280} and A_{295} of the extracts were also determined.

6.4.3.6 Antioxidative activities

Water extracts prepared as mentioned above were determined for antioxidative activities. Prior to assay, the extracts were approximately diluted using distilled water.

- DPPH radical scavenging activity

DPPH radical scavenging activity was determined according to the method of Faithong and Benjakul (2012) with a slight modification. The standard curve was prepared using Trolox in the range of 10-60 μM. The activity was expressed as μmol Trolox equivalents (TE)/g sample.

- ABTS radical scavenging activity

ABTS radical scavenging activity was determined as described by Sun and Tanumihardjo (2007) with a slight modification. A Trolox standard curve (50-600 μM) was prepared and ABTS radical scavenging activity was expressed as μmol TE/g sample.

- Ferric reducing antioxidant power

Ferric reducing antioxidant power (FRAP) was evaluated by the method of Benzie and Strain (1996). The standard curve was prepared using Trolox ranging from 50 to 600 μM. The activity was expressed as μmol TE/g sample.

6.4.3.7 Volatile compounds

Volatile compounds of *Kapi* samples were determined using a solid-phase microextraction gas chromatography mass spectrometry (SPME GC-MS)

following the method of Pongsetkul *et al.* (2014). The identified volatile compounds were expressed in the terms of relative abundance.

6.4.3.8 Sensory property

Kapi samples were evaluated by 50 untrained panellists, who consumed *Kapi* regularly. The samples were wrapped with aluminum foil and heated in hot air oven at 60°C for 30 min. Samples were cut to obtain a thickness of 1 cm (2×2 cm^2), then served in white paper plate at room temperature. Panellists were instructed to rinse their mouths with water, cucumber or cracker between different samples. The panellists were asked to assess samples for appearance, color, odor, flavor, texture and overall-liking using a 9-point hedonic scale (Mellgard *et al.,* 2007).

6.4.4 Statistical analysis

All experiments were conducted in triplicate. Statistical analysis was performed using one-way analysis of variance. Mean comparison was carried out using Duncan's multiple range test (Steel *et al.,* 1980). SPSS statistic program (SPSS 10.0 for windows, SPSS Inc., Chicago, IL) was used for data analysis.

6.5 Results and discussion

6.5.1 Quality changes of shrimp during postmortem storage

6.5.1.1 Changes in pH

A slight increase in pH of shrimp (*M. lanchesteri*) from 7.07 to 7.42 was observed as the post-mortem time increased (Figure 14). The increase of pH might be associated with the production of volatile basic components, such as ammonia, trimethylamine, etc. by some spoilage bacteria (Pongsetkul *et al.,* 2014). However, the different buffering capacity of muscle proteins from different species plausibly contributed to varying rate of pH changes (Riebroy *et al.,* 2008). In the present study, shrimp were stored at room temperature, in which the deterioration could take place to a faster rate, compared with the storage in ice or refrigerated condition. After capture,

shrimp were placed in container without icing before off-loading. This could induce the spoilage, particularly with the longer storage or handling time.

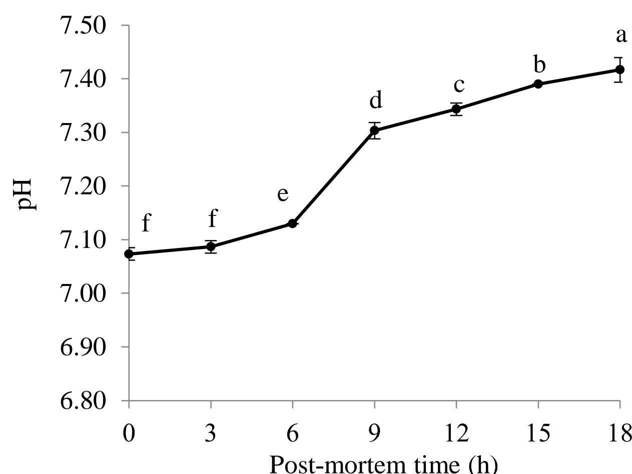

Figure 14. pH of shrimp *M. lanchesteri* during post-mortem storage at room temperature.

Bars represent the standard deviation from triplicate determinations. Different lowercase letters on the bars indicate the significant difference ($p < 0.05$).

6.5.1.2 Changes in TVB and TMA contents

Figure15A and Figure15B show the changes in TVB and TMA contents of shrimp during 18 h of post-mortem storage at room temperature. TVB and TMA contents of fresh shrimp (0 h) was 6.99 mg N/100 g sample and 4.70 mg N/100 g sample, respectively. As the post-mortem time increased, both TVB and TMA content continuously increased up to the end of storage (18 h) ($p < 0.05$). After 18 h, the highest TVB (81.55 mg N/100 g sample) and TMA content (14.49 mg N/100 g sample) were obtained. This was more likely the results of the deterioration of nitrogenous compounds. Trimethylamine oxide (TMAO), a non-volatile and non-odouriferous compound, could be reduced to trimethylamine (TMA) as mediated by spoilage microorganisms (Dissaraphong *et al.,* 2006). In general, the increases in both TVB and TMA contents were in accordance with the increase in pH (Figure 1A). The result suggested that the spoilage caused by bacteria occurred to a higher extent in shrimp, particularly when the storage time increased.

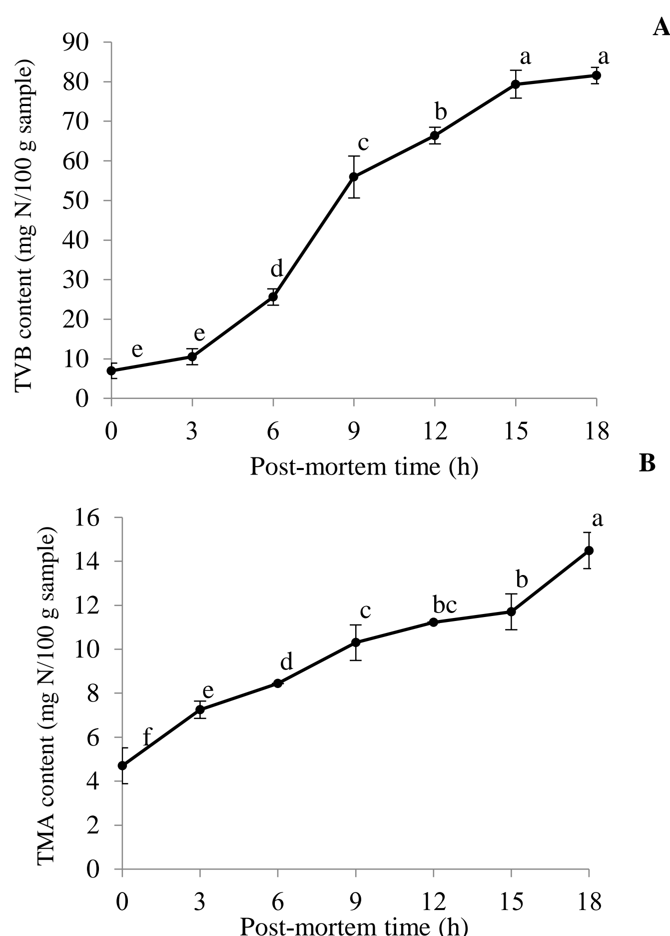

Figure 15. Total volatile base (TVB) (A) and trimethylamine (TMA) contents (B) of shrimp *M. lanchesteri* during post-mortem storage at room temperature.

Bars represent the standard deviation from triplicate determinations. Different lowercase letters on the bars indicate the significant difference ($p < 0.05$).

6.5.1.3 Changes in TBARS

Changes in TBARS value of shrimp during 18 h of post-mortem storage at room temperature are presented in Figure 16A. TBARS value of fresh shrimp was 0.60 mg MDA/kg sample. It was increased when post-mortem time increased ($p < 0.05$) and the highest TBARS value (0.97 mg MDA/kg sample) was found in shrimp with post-mortem time of 18 h ($p < 0.05$). The result suggested that lipid oxidation took place during the extended storage. Shrimp or krill lipids have been known to contain high content of polyunsaturated fatty acid (PUFA) (Takeungwongtrakul *et al.,* 2012). Those PUFA are prone to oxidation as indicated by the presence of TBARS in the samples. Furthermore, autolysis caused by endogenous proteases might lead to the disruption of

the organelles, thereby facilitating the release of pro-oxidants as well as reactants (Dissaraphong *et al.,* 2006). This led to the enhanced lipid oxidation in the sample.

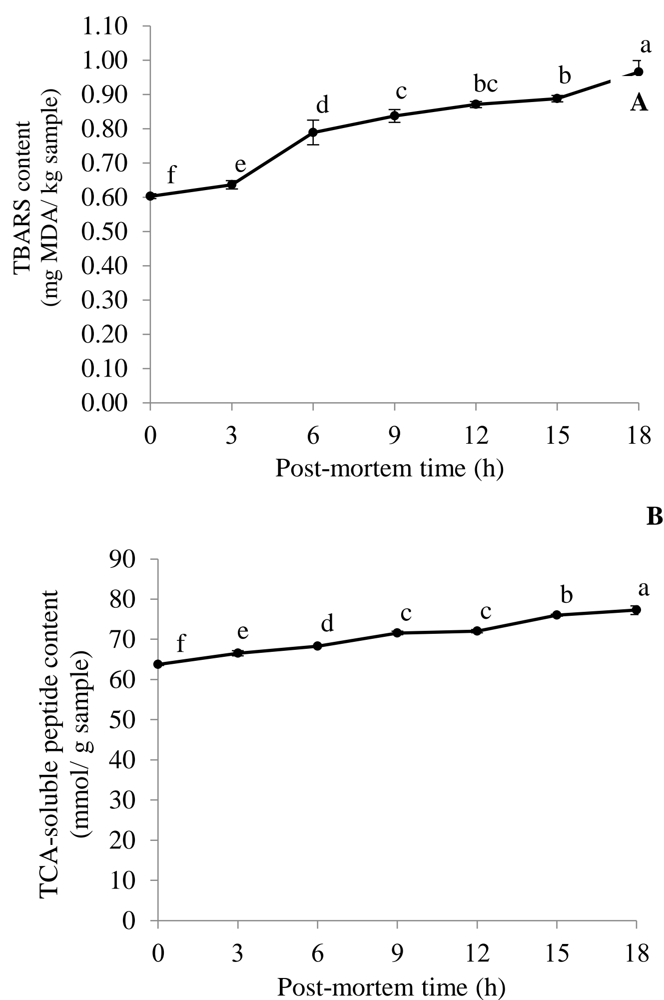

Figure 16. Thiobarbituric acid reactive substances (TBARS) (A) and TCA-soluble peptide contents (B) of shrimp *M. lanchesteri* during post-mortem storage at room temperature.

Bars represent the standard deviation from triplicate determinations. Different lowercase letters on the bars indicate the significant difference ($p < 0.05$).

6.5.1.4 Changes in TCA-soluble peptide

TCA-soluble peptide content of fresh shrimp (0 h) was 63.75 mmol/g sample (Figure 16B). It was suggested that protein of shrimp rapidly degraded into small peptide or free amino acids after death, particularly during transportation. As the post-mortem time increased, a continuous increase in oligopeptides was observed ($p < 0.05$). After 18 h, TCA-soluble peptide content of shrimp was 77.26 mmol/g sample. Whole shrimp contained the cephalothorax, where hepatopancreas and other digestive organs were located. Hepatopancreas of Pacific white shrimp was rich in trypsin (Senphan and Benjakul, 2014). Therefore, degradation caused by both indigenous and microbial proteases could be enhanced when shrimp were stored at room temperature for an extended time as evidenced by the higher TCA-soluble peptide contents.

6.5.1.5 Protein patterns

Figure 17 shows protein patterns of whole shrimp with various post-mortem storage times. Fresh shrimp contained myosin heavy chain (MHC) as the most dominant protein. The band intensity of MHC was gradually decreased when post-mortem storage time increased. MHC still remained at 10-15%, compared to that found in fresh shrimp (0 h), after storage time for 18 h. Sriket *et al.* (2012) reported that MHC is susceptible to proteolytic degradation than other muscle proteins such as actin, troponin and tropomyosin. For actin (MW of 45 kDa), it was found at lower extent, compared with MHC. It was noted that band intensity of actin remained constant throughout 18 h of storage. Additionally, proteins or peptides with MW about 100 kDa continuously decreased, whilst proteins or peptides with MW about 20 kDa increased with increasing storage time. The result suggested that proteins in shrimp underwent degradation drastically during post-mortem storage. Shrimp cephalothorax containing hepatopancreas has been known to be the major source of proteases, mainly serine protease and metalloprotease (Sriket *et al.,* 2012). Those indigenous proteases along with bacterial proteases played a role in hydrolysis of proteins in shrimp. The formation of low molecular weight peptides was in accordance with the increasing TCA-soluble peptide contents (Figure 16B).

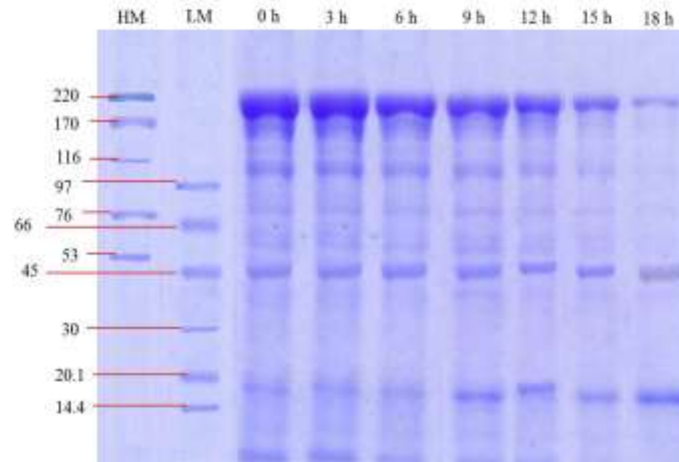

Figure 17. Protein patterns of shrimp *M. lanchesteri* during post-mortem storage at room temperature. HM: high molecular weight marker, LM: low molecular weight marker.

6.5.1.6 Change in TVC

Fresh shrimp showed initially TVC of 3.22 log colony forming units (CFU)/g sample (Figure 18). Microbiological counts of shrimp increased as post-mortem time increased ($p < 0.05$). After 18 h of storage at room temperature, the highest TVC (5.75 log CFU/g sample) was found. In general, TVC increased markedly when the sample was stored at room temperature, in which mesophiles could grow rapidly. Vanderzant *et al.* (1973) reported that warm water marine shrimp often showed total aerobic counts of 10^6 CFU/g sample when captured, but after cleaning process and storage at low temperature, microbial count can be lowered. Those microorganisms might contribute to the final quality of the resulting *Kapi* when used as raw material.

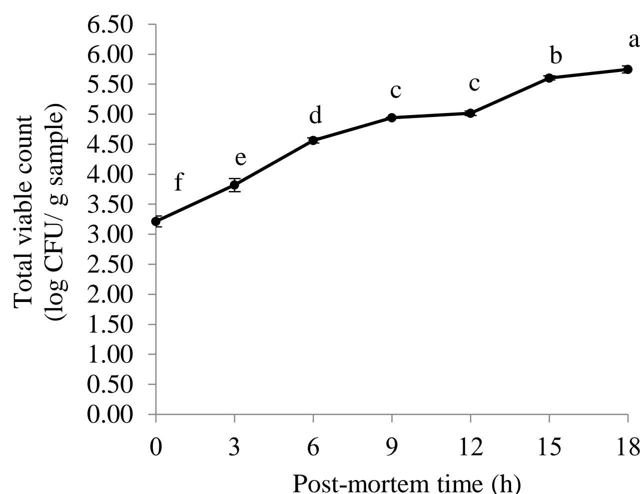

Figure 18. Total viable count (TVC) of shrimp *M. lanchesteri* during post-mortem storage at room temperature.

Bars represent the standard deviation from triplicate determinations. Different lowercase letters on the bars indicate the significant difference ($p < 0.05$).

6.5.2 Effect of postmortem storage time on characteristics and properties of *Kapi*

6.5.2.1 pH

pHs of *Kapi* produced from shrimp with different post-mortem storage times are shown in Table 19. pH of all samples was in ranges of 7.28-7.54. There were no differences in pH ($p > 0.05$) amongst *Kapi* samples produced from shrimp stored for up to 12 h. Nevertheless, *Kapi* prepared from shrimp stored for 18 h had the highest pH (7.54) ($p < 0.05$). Higher pH of *Kapi* correlated with the increasing pH of shrimp used as raw material, particularly those having the longer post-mortem storage time. This was also in accordance with increasing TVB content in raw material (Figure 15A).

Table 19. pH, color, browning intensity and antioxidative properties of *Kapi* produced from shrimp *M. lanchesteri* with different post-mortem times

Properties	Post-mortem time			
	0 h	6 h	12 h	18 h
pH	7.28±0.53[b]	7.31±0.10[b]	7.30±0.35[b]	7.54±0.46[a]
*L**	49.76±0.30[a]	45.95±1.01[b]	42.94±0.46[c]	41.76±1.07[d]
*a**	6.53±0.27[c]	6.89±0.30[b]	7.67±0.18[a]	7.67±0.93[a]
*b**	13.54±0.49[a]	11.88±0.29[b]	11.41±0.17[c]	9.36±0.45[d]
Δ*E**	46.32±0.41[c]	49.58±0.91[b]	52.50±0.43[a]	53.25±1.14[a]
Δ*C**	14.12±0.53[a]	12.82±0.22[b]	12.83±0.06[b]	11.19±0.50[c]
A_{280}	0.64±0.02[c]	1.04±0.07[a]	0.92±0.05[b]	1.06±0.12[a]
A_{295}	0.78±0.01[c]	0.77±0.19[d]	0.84±0.11[b]	0.87±0.06[a]
Browning intensity (A_{420})	0.15±0.03[d]	0.22±0.04[c]	0.35±0.02[b]	0.56±0.01[a]
Fluorescence intensity	392.67±8.47[b]	399.34±6.66[b]	397.22±6.76[b]	416.85±1.90[a]
DPPH radical scavenging activity (µmol TE/ g sample)	1.05±0.19[d]	1.65±0.19[c]	2.14±0.14[b]	2.38±0.19[a]
ABTS radical scavenging activity (µmol TE/ g sample)	10.22±0.13[d]	12.06±0.17[c]	13.69±0.13[b]	15.62±0.21[a]
FRAP (µmol TE/ g sample)	7.59±0.31[d]	10.61±0.25[c]	11.85±0.18[b]	12.89±0.24[a]

Values in parentheses indicate the content expressed, based on dry weight.
Mean ± SD from triplicate determinations. Different lowercase superscripts in the same row indicate the significant difference ($p < 0.05$).

6.5.2.2 Nitrogen content

Formal, ammonia and amino nitrogen contents of resulting *Kapi* are depicted in Figure 19. Formal nitrogen content increased from 12.21 to 16.61 mg N/g sample as post-mortem time of shrimp used as raw material increased from 0 to 18 h. In general, formal nitrogen content has been used to measure the degree of protein hydrolysis (Faithong and Benjakul, 2012). The results indicated that shrimp having the longer post-mortem storage time yielded *Kapi* with the greater degradation of protein as evidenced by greater formal nitrogen content. The varying degradation of proteins might contribute to characteristics of *Kapi* differently.

Ammonia nitrogen content of all *Kapi* samples increased when the post-mortem time of shrimp used as raw material increased ($p < 0.05$) as shown in Figure 19. *Kapi* produced from shrimp with 18 h of post-mortem time showed the highest ammonia nitrogen content (1.75 mg N/g sample). The ammonia nitrogen content indicates the breakdown of soluble protein and peptides into free amino acid and volatile nitrogen (Faithong and Benjakul, 2012). Higher degradation of protein might

favour the subsequent deamination of proteins, as indicated by higher ammonia nitrogen content. Amino nitrogen contents in *Kapi* using shrimp having various post-mortem times as raw material are depicted in Figure 19. Amino nitrogen content represents the amount of primary amino group of the sample. An increase in amino nitrogen content is related to the degradation of polypeptide (Pongsetkul *et al.,* 2014). A similar trend was found to that of formal and ammonia nitrogen contents. The results suggested that the longer post-mortem storage times of raw material resulted in considerable increases in free amino acids. Those free amino acids might serve as the nutrient for microorganisms. Also they could contribute to the taste or flavour of *Kapi.*

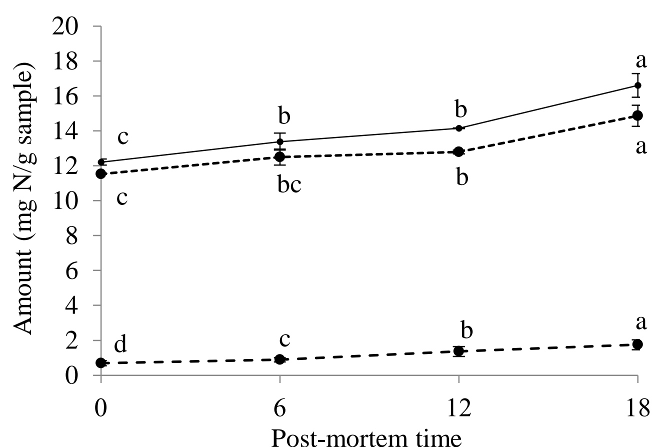

Figure 19. Formal nitrogen content (———), ammonia nitrogen content (--------) and amino nitrogen content (-------) of *Kapi* produced from shrimp *M. lanchesteri* with different post-mortem times.

Bars represent the standard deviation from triplicate determinations. Different lowercase letters on the bars indicate the significant difference ($p < 0.05$).

6.5.2.3 Color

Kapi produced from shrimp with different post-mortem storage times had differences in colour as shown in Table 19. L^* (lightness), a^* (redness), b^* (yellowness), ΔE^* (total difference in color) and ΔC^* (difference in chroma) were in the range of 41.76-59.76, 6.53-7.67, 9.36-13.54, 46.32-53.25 and 11.19-14.12, respectively. L^*, b^* and ΔC^*-value of *Kapi* decreased when the post-mortem time of shrimp used as raw material increased, whereas a^*, ΔE^*-value of *Kapi* sample slightly

increased with increasing post-mortem times of shrimp prior to salting ($p < 0.05$). The results indicated that *Kapi* became darker when unfresh shrimp were used as raw material. *Kapi* produced from shrimp with 18 h of post-mortem time had the highest a^*-value, suggesting that the pronounced autolysis might cause the increased release of carotenoids from carotenoprotein during extended post-mortem times of shrimp. Astaxanthin, has a red-orange in colour, especially when it was separated from protein moiety (Faithong and Benjakul, 2012). With the delay in salting, biochemical change, especially enzymatic reactions e.g. polyphenoloxidase (PPO) occurred (Nirmal and Benjakul, 2009). This more likely contributed to the darker colour of resulting *Kapi.*

6.5.2.4 Browning and Maillard reaction product

Browning intensity and Maillard reaction product of water extract of *Kapi* produced from shrimp with different post-mortem storage times are shown in Table 19. Browning intensity (A_{420}) of resulting *Kapi* was observed when there was the delay in salting of shrimp ($p < 0.05$). During extended post-mortem times, enzymatic browning reactions induced by polyphenoloxidase (PPO) occurred (Pongsetkul *et al.,* 2014). PPO has been known to induce the hydroxylation of phenols with subsequent polymerisation, in which melanin is formed (Nirmal and Benjakul, 2009). Therefore, prolonged post-mortem times prior to salting might result in substantial increases in browning intensity. This coincided with the decreases in L^*-value (Table 19).

A_{280} and A_{295} of resulting *Kapi* slightly increased when the post-mortem time of shrimp used as raw material increased ($p < 0.05$). Generally, A_{280} and A_{295} have been used to determine the formation of non-fluorescent intermediate compounds of the Maillard reaction (Ajandouz *et al.,* 2001). With larger amount of free amino group in shrimp stored for a longer time, the Maillard reaction could take place during fermentation of 30 days to a higher degree. This was evidenced by higher A_{280} and A_{295}.

No differences in fluorescence intensity among *Kapi* produced from shrimp stored for 0, 6 and 12 h ($p > 0.05$). Nevertheless, the increase in fluorescence intensity was noticeable in *Kapi* produced from 18 h-stored shrimp. Fluorescence intensity has been used to monitor the occurrence of intermediate products, which subsequently undergo polymerisation to form the brown pigments (Ajandouz *et al.,* 2001). With higher Maillard reaction intermediates, *Kapi* produced from shrimp with

18 h of post-mortem time could further undergo browning reaction to a higher extent. As a result, *Kapi* was browner in color.

6.5.2.5 Antioxidative activities

Antioxidant activities of water extract of *Kapi* produced from shrimp with different post-mortem times as tested by DPPH, ABTS radical scavenging activity and FRAP are presented in Table 19. DPPH, ABTS radical scavenging activity and FRAP of water extracts of *Kapi* were in range of 1.05-2.38, 10.22-15.62 and 7.59-12.89 μmol TE/g sample, respectively. ABTS radical scavenging assay has been used to determine both hydrophilic and lipophilic antioxidants. FRAP is generally used to measure the capacity of a substance in reducing TPTZ-Fe(III) complex to TPTZ-Fe(II) complex (Sun and Tanumihardjo, 2007). Overall, DPPH, ABTS radical scavenging activity and FRAP of water extracts of *Kapi* increased when the post-mortem time of shrimp used as raw material for *Kapi* production increased ($p < 0.05$). Water extract of *Kapi* produced from shrimp with 18 h of post-mortem time showed the highest DPPH, ABTS radical scavenging activity and FRAP. On the other hand, the extract of *Kapi* produced by fresh shrimp (0 h) had the lowest activities. During the extended post-mortem storage prior to salting, low molecular weight peptides and amino acids could be generated to a higher extent. Protein hydrolysis or degradation during fermentation step also led to the formation of substantial active peptides which could interact with free radical and terminate the chain reaction of auto-oxidation. This was correlated with pronounced degradation of proteins in shrimp stored for a longer time before salting (Figure 17). The extracts from salted shrimp paste have been reported to possess antioxidant activities (Pongsetkul *et al.,* 2015). Furthermore, Maillard reaction products might be partially involved in antioxidant activity. Maillard reaction products were reported to have antioxidative activity (Benjakul *et al.,* 2005). Maillard reaction products were also increased in *Kapi* prepared from unfresh (Table 19). Therefore, antioxidative activity of *Kapi* was governed by post-mortem time of shrimp prior to salting.

6.5.2.6 Volatile compounds

Volatile compounds in *Kapi* produced from shrimp with different post-mortem times analysed by SPME GC-MS are shown in Table 20. Thirty-six volatile compounds were isolated and identified. Those compounds could be classified as aldehydes (5), ketones (6), alcohols (9), nitrogen-containing compounds (7), hydrocarbons (4) and others (5). Lipid-derived components such as aldehydes, alcohols as well as nitrogen-containing compounds were the major volatile compounds in *Kapi.*

3-methyl-butanal, pentanal, 4-heptanal, hexanal and benzaldehyde were the prevalent aldehydes found in all *Kapi* samples. *Kapi* with varying abundance of aldehydes might influence the flavour acceptability differently. In general, the presence of these compounds is related with lipid oxidation, which was more likely generated during storage or fermentation (Dissaraphong *et al.,* 2006). Shrimp contained high amounts of ω-3 fatty acids, which were highly susceptible to lipid oxidation (Takeungwongtrakul *et al.,* 2012). *Kapi* produced from shrimp stored for 18 h before salting showed higher abundance in all aldehydes, except 4-hexanal. This was coincidental with the highest TBARS value of shrimp used as raw material stored for 18 h (Figure 16A). Furthermore, Steinhaus and Schieberle (2007) reported that branched short chain aldehydes or aromatic aldehydes plausibly resulted from deamination of amino acids. This was also in agreement with the highest amino nitrogen content found in this sample (Figure 19).

Ketones were also found in *Kapi* including 2-heptanone, 1-(2-pyridinyl)-ethanone, 3,5-Octadien-2-one, etc. The higher abundance of most ketone compounds was obtained in *Kapi* produced from shrimp with 18 h of post-mortem time. This confirmed that delayed post-mortem salting allowed more lipid oxidation in raw material to take place. Those products were still presented in resulting *Kapi.* However, such compounds with low concentrations and high odour threshold values might not significantly contribute to flavour of salted shrimp paste (Cha and Cadwallader, 1995).

Table 20. Volatile compounds of *Kapi* produced from shrimp *M. lanchesteri* with different post-mortem times

Volatile compounds	Peak area (Abundance)×10^6			
	0 h	6 h	12 h	18 h
Aldehydes				
3-methyl-butanal	215.22	206.11	411.12	732.51
Pentanal	294.58	199.76	408.81	466.25
4-heptanal	117.22	132.99	130.19	98.66
Hexanal	54.51	501.11	511.19	526.63
Benzaldehyde	162.8	203.42	452.61	688.22
Ketones				
1-phenyl-ethanone	128.71	134.34	205.55	205.13
1-(2-pyridinyl)-ethanone	237.93	304.49	314.51	505.82
1-(2-aminophenyl)-ethanone	150.54	68.88	102.25	111.33
2-Heptanone	597.82	413.22	499.65	404.52
2-Octanone	265.60	244.12	300.64	305.77
3,5-Octadien-2-one	117.14	332.26	304.55	502.65
Alcohols				
Benzenemethanol	42.95	35.55	69.32	167.83
2-Butoxy-ethanol	56.14	181.22	555.92	608.73
3-methyl, 1-butanol	299.19	625.55	632.72	820.24
1-Pentanol	225.61	203.44	144.41	28.32
1-Penten-3-ol	188.13	55.67	59.11	9.88
1-Hexanol	404.33	133.92	129.29	266.51
2-ethyl-hexanol	122.97	166.92	208.82	277.01
1-Octen-3-ol	171.89	204.45	229.33	301.99
Octa-1,5-dien-3-ol	57.04	ND	106.66	169.72
Nitrogen-containing compounds				
Methyl-pyrazine	214.34	506.66	511.23	495.95
Trimethyl-pyrazine	252.17	345.52	304.41	406.77
2,5-dimethyl-pyrazine	898.28	933.49	925.67	1055.61
2,6-dimethyl-pyrazine	90.42	215.62	307.66	313.49
2-ethyl-5-methyl-pyrazine	1524.77	1866.17	1908.22	1855.52
2-ethyl-2,5-dimethyl-pyrazine	155.54	260.08	477.71	503.31
3-ethyl-2,5-dimethyl-pyrazine	644.91	601.98	802.22	899.13
Hydrocarbon				
2,6,10,14-tetramethyl-pentadecane	21.53	ND	19.22	111.19
2-Undecyne	ND	13.31	455.63	109.92
Hexadecane	ND	ND	61.63	13.34
Cyclododecane	23.98	50.66	45.61	77.84
Others				
2-methyl-propanoic acid	68.42	56.33	ND	23.18
Octanoic acid	126.10	15.03	ND	102.31
Methyl-ester-octadecanoic acid	ND	19.44	18.49	ND
Phenol	261.08	505.55	635.48	904.41
1H-Indole	711.41	805.92	8908.23	9181.46

ND: Not detectable.

Normal and branched alcohols, which were quite low in abundance, also detected in *Kapi*. 3-methyl, 1-butanol was found at higher abundance than others. This alcohol was increased in *Kapi* prepared from shrimp with increasing post-mortem times. However, Cha and Cadwallader (1995) reported that alcohols might not have a paramount impact on *Kapi* flavor because of their high flavor thresholds.

All resulting *Kapi* consisted of 7 nitrogen-containing compounds, which were all pyrazine derivatives. 2-ethyl-5-methyl-pyrazine was dominant in all samples, followed by 2,5-dimethyl-pyrazine and 3-ethyl-2,5-dimethyl-pyrazine. Jaffres *et al.* (2011) reported that pyrazines derivatives associated with meaty flavour of shrimp sauce. Abundance of most pyrazine derivatives of resulting *Kapi* increased when the post-mortem time of shrimp used as raw material increased ($p < 0.05$). Pyrazines were reported to be formed by Maillard reaction through strecker degradations from various nitrogen sources such as amino acids (Cha and Cadwallader, 1995). This was in accordance with increasing browning intensity when post-mortem time of shrimp was extended. Thus, different freshness of raw material more likely had the influence on meaty flavour, as well as browning colour to some degrees.

Four hydrocarbon compounds were found in *Kapi.* Only cyclododecane was detected in all *Kapi. Kapi* produced from shrimp with 18 h of post-mortem time showed the higher abundancre than others. Alkanes and alkenes are mainly formed from lipid autooxidation of fatty acids released from triglycerides (Latorre-Moratalla *et al.,* 2011).

Additionally, all samples contained phenol and indole and *Kapi* produced from shrimp with 18 h of post-mortem times had the highest abundance than other samples. Cha and Cadwallader (1995) reported that phenol give an undesirable aroma in seafoods, whilst Lakshmanan *et al.* (2002) reported that indole is the degradation product from tryptophan and has been used as the index for shrimp spoilage. The higher abundance of indole in *Kapi* correlated well with the increased spoilage of shrimp as indicated by increased TVB, TMA as well as TVC.

Therefore, several reactions might be involved in formation of volatiles. Lipid hydrolysis and autooxidation, proteolysis and transformation of amino acids to several compounds could be generated during *Kapi* production. *Kapi* produced from shrimp with different post-mortem times showed varying volatile compounds. Thus,

post-mortem times of shrimp used as raw material might have a great influence on the volatile composition which impact on the final flavor and odor of resulting *Kapi.*

6.5.2.7 Sensory properties

Likeness scores of *Kapi* produced from shrimp *M, lanchesteri* with different post-mortem times are shown in Table 21. Amongst all samples, appearance, color and texture likeness scores were not different ($p > 0.05$). However, *Kapi* produced from shrimp with 18 h of post-mortem time showed the lowest odour and flavour likeness score ($p < 0.05$). The result suggested that this sample might have undesirable volatiles, which were associated with deterioration products, particularly formed during the extended post-mortem storage prior to salting. This was in agreement with the highest abundance of aldehydes, ketones, phenol along with indole found in this sample. Furthermore, *Kapi* produced from shrimp stored for 18 h prior to salting also had the lowest overall likeness score ($p < 0.05$). Different tastes or flavors were possibly caused by differences in volatile compounds (Table 20), governed by freshness of raw material used. It was noted that *Kapi* prepared from shrimp stored for 6 h prior to salting showed the highest overall likeness score ($p < 0.05$). This might be caused by partial decomposition of nucleotides, in which some derivatives, especially inosine monophosphate (IMP) was formed. IMP has been known to have the umami taste (Hajeb and jinap, 2015). Non-significantly higher score for flavor likeness was also found in this sample. Based on overall likeness score, shrimp should not be stored more than 12 h before salting.

Table 21. Likeness score of *Kapi* produced from shrimp with different post-mortem times

Attributes	Post-mortem time			
	0 h	6 h	12 h	18 h
Appearance	6.86±0.77^{a}	6.81±0.93^{a}	6.92±0.87^{a}	6.95±0.74^{a}
Color	6.90±0.83^{a}	7.05±0.92^{a}	7.24±0.83^{a}	7.14±0.91^{a}
Odor	6.90±0.97^{ab}	7.38±1.02^{a}	6.52±0.93^{b}	5.81±1.08^{c}
Texture	6.95±0.80^{a}	7.00±0.95^{a}	7.05±0.97^{a}	6.57±0.68^{a}
Flavor	6.67±1.08^{ab}	7.10±0.70^{a}	6.38±0.74^{b}	5.48±1.02^{c}
Overall	6.57±0.68^{b}	7.29±0.96^{a}	6.19±1.27^{bc}	5.86±1.02^{c}

Values are given as mean ± SD (n = 3). Different lowercase superscripts within the same row indicate the significant differences ($p < 0.05$).

6.6 Conclusion

Small shrimp *M. lanchesteri* could be used as an alternative raw material for *Kapi* production. Protein degradation as well as lipid oxidation proceeded during the extended storage. Freshness of shrimp affected *Kapi* characteristics, in which *Kapi* became darker but had higher antioxidative activity as the raw material was unfresh. For this shrimp species, *Kapi* produced from shrimp with post-mortem time of 6 h had the highest overall-liking score, compared with others. However, the delay in salting of shrimp should not exceed 12 h, when the resulting *Kapi* still had the sensorial property equivalent to that prepared from fresh shrimp.

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CHAPTER 7

COMPARATIVE STUDIES ON AUTOLYSIS AND ANTIOXIDATIVE PROPERTIES OF SALTED SHRIMP PASTE (*KAPI*) FROM *ACETES VULGARIS* AND *MACROBRACHIUM LANCHESTERI*

7.1 Abstract

Autolysis of *Acetes vulgaris* and *Macrobrachium lanchesteri* was comparatively studied. Maximal autolytic activities were found at pH 7, 55°C for *A. vulgaris* and pH 8, 60°C for *M. lanchesteri.* Both shrimps dominantly contained serine proteases as indicated by the marked inhibition by PMSF and SBTI. When both shrimps were used for *Kapi* production and characterized, differences in characteristics as well as antioxidant activities were obtained. *Kapi* produced from *A. vulgaris* (KA) showed the higher degree of hydrolysis (15.93±0.51%), peroxide values (0.20±0.23 mg cumene peroxide/kg sample) and TBARS value (0.55±0.16 mg MDA/kg sample), compared with *Kapi* produced from *M. lanchesteri* (KM), indicating the higher protein degradation and lipid oxidation in this sample. Moreover, KA also possessed the higher antioxidative activities including ferric reducing antioxidant power (FRAP) (14.12±1.34 μmol TE/g sample), chelating activity (3.21±0.05 μmol EE/g sample), hydrogen peroxide (30.05±0.49 μmol TE/g sample) and singlet scavenging activities (48.46±0.32 μmol EE/g sample) than KM, except for DPPH and ABTS radical scavenging activities, which showed lower activity ($p < 0.05$) and had no differences ($p > 0.05$), respectively Therefore, different levels and types of endogenous proteases in both shrimps determined the autolysis and characteristics as well as antioxidative properties of resulting *Kapi*.

7.2 Introduction

Kapi, a traditional salted shrimp paste, has been consumed widely in Thailand as a condiment to enhance the palatability of foods in various dishes. *Kapi* has a strong typical flavor and its color varies from a pinkish or purplish grey to a dark

greyish brown (Pongsetkul *et al.,* 2014). *Kapi* is made by mixing shrimp or krill with solar salt at a ratio of 3-5: 1. After being salted, the mixture is sun-dried, and then it is thoroughly blended or homogenized to produce semi-solid paste. The paste is fermented at room temperature (25-30°C) for at least 1 month or longer until the typical aroma is developed (Faithong *et al.,* 2010). Nowadays, the planktonous krill (*Mesopodopsis orientalis*), which is the traditional raw material for *Kapi* production, has dropped by 3% per year over the last decade (Meland and Willassen, 2007). To conquer the insufficient raw material, the alternative source, especially small shrimp belonging to *Acetes vulgaris*, has become promising for *Kapi* production in the southern part of Thailand, owing to its high availability (Faithong *et al.,* 2010). Recently, another species, *Macrobrachium lanchesteri*, a by-catch from commercial fishing, has been used as an alternative raw material because of its abundance, lower price, and availability throughout the year (Pongsetkul *et al.,* 2016a). For *Kapi* production, differences in raw material, the quantity of salt used, and the treatment of raw materials prior to fermentation result in varying compositions and quality of final products (Peralta *et al.,* 2008). Pongsetkul *et al.* (2015a) found that the delay before salting led to the distorted quality of *Kapi* due to microbial deterioration. It has been known that autolysis, which varies with shrimp species, is a crucial process in converting raw material to final *Kapi.*

Kapi has been reported to contain peptides with bioactivity, especially antioxidant activity (Peralta *et al.,* 2008; Faithong and Benjakul, 2012; Pongsetkul *et al.,* 2014). Cleavage of food proteins by microbial or indigenous proteases yields the bioactive peptides, leading to substantial increases in the biological properties of fermented food products (Faithong *et al.,* 2010). *Kapi* produced from different shrimps, mediated by different indigenous proteases, might have different characteristics and antioxidative activities. Nevertheless, no information on autolysis of shrimp, *A. vulgaris* and *M. lanchesteri,* and properties of resulting *Kapi* from both species, has been reported.

7.3 Objective

To comparatively study the autolysis of both shrimp species and to characterize some properties and antioxidative activities of *Kapi* produced from both shrimps.

7.4 Materials and methods

7.4.1 Sample collection

For each lot, 10 kilograms of shrimp *A. vulgaris* (average body length 15.6±1.4 mm, average wet weight 0.0413±0.0098 g, n=20) and *M. lanchesteri* (average body length 27.5±1.9 mm, average wet weight 0.0701±0.0107 g, n=20) were caught from the coast in Ko-yo and The-Pha in Songkhla province, Thailand, respectively. After capture, shrimp were transported in ice with a shrimp/ice ratio of 1:2 (w/w) in a polystyrene container to the Department of Food Technology, Prince of Songkla University, Hat Yai, Thailand, within approximately 2 h.

7.4.2 Study on autolysis of *A. vulgaris* and *M. lanchesteri*

7.4.2.1 Effect of temperature

Whole shrimps with the physiological pH of 7.0-7.4 were ground using a blender. Ground shrimp (3 g) was placed in a 50-ml beaker and covered with aluminium foil. The samples were incubated for 60 min at various temperatures (30-80°C) in a temperature-controlled water bath (Memmert, Schwabach, Germany). Autolytic reaction was terminated by adding 27 ml of cold 5% (w/v) trichloroacetic acid (TCA). The mixture was homogenized using an IKA Labortechnik homogenizer (Selangor, Malaysia) at a speed of 11,000 rpm for 2 min, followed by centrifugation at 8,000×*g* for 30 min. The amount of the soluble peptide in the supernatant was measured using the Lowry method (Lowry *et al.,* 1951) and expressed as mmol tyrosine equivalent/min/g sample.

7.4.2.2 Effect of pH

Ground shrimp (3 g) was homogenized at 11,000 rpm for 1 min with 12 ml of different buffers having various pHs (McIlvain's buffer consisting of 0.2 M sodium phosphate and 0.1 M sodium citrate with pH range of 2-7, and 0.1 M glycine-NaOH with pH range of 8-11). The homogenate from each species was incubated at the corresponding temperature. Autolysis was terminated after 60 min of incubation by the addition of 15 ml of cold 9% (w/v) TCA, followed by centrifugation as previously described. Soluble peptides in the supernatant were determined using the Lowry method and expressed as mmol tyrosine equivalent/min/g sample.

7.4.2.3 Effect of salt concentration

The effect of NaCl at various concentrations on autolytic activity was studied. Firstly, the buffer showing the maximal activity was added with NaCl to obtain different final concentrations [0, 5, 10, 15, 20, 25 and 30% (w/w)]. Ground sample (3 g) was mixed with the aforementioned buffers and then incubated at the optimal temperature for 60 min. After incubation, 15 ml of cold 9% (w/v) TCA were added and the mixture was homogenized (Klomklao *et al.,* 2008). Soluble peptides were determined by using the Lowry method (Lowry *et al.,* 1951) and expressed as mmol tyrosine equivalent/min/g sample.

7.4.2.4 Effect of various protease inhibitors

Ground shrimp (0.5 g) was homogenized (11,000 rpm for 1 min) with 1.5 ml of the buffer yielding the maximal activity. The homogenate was mixed with 2 ml of protease inhibitor solution to obtain the final designated concentrations (1 mM pepstatin A, 0.1 mM E-64, 10 mM EDTA, 1 g/l SBTI, 5 mM PMSF, 5 mM TLCK, 5 mM TPCK and 1 mM Iodoacetic acid) (Sriket *et al.,* 2011). The mixtures were allowed to stand in ice for 30 min, followed by incubation at the optimal temperature for 60 min. Autolysis was terminated by addition of 2 ml of of cold 15% (w/v) TCA. The mixture was homogenized and centrifuged as mentioned above. The soluble peptide contents were then determined by using the Lowry method (Lowry *et al.,* 1951) and expressed as mmol tyrosine equivalent/min/g sample.

7.4.3 Study on properties of *Kapi* produced from *A. vulgaris* and *M. lanchesteri*

7.4.3.1 Preparation of *Kapi*

Kapi was prepared following the method of Pongsetkul *et al.* (2015a). Shrimps were mixed with salt at the ratio of 5:1 (w/w) and then kept at room temperature (28-32°C) overnight. Subsequently, the samples were drained, mashed, spread out on fiberglass mats and dried with sunlight (38-42°C). After the moisture contents of dried shrimp were in the range of 35-40%, salted shrimps were transferred into earthen jars and covered with plastic bag tightly (close system). After 30 days of fermentation at room temperature (28-32°C), *Kapi* samples were collected and referred to as KA (*Kapi* produced from *A. vulgaris*) and KM (*Kapi* produced from *M. lanchesteri*). The obtained samples were subjected to analyses.

7.4.3.2 Formal, ammonia and amino nitrogen contents

Formal nitrogen content was determined by the titration method as described by Thai Industrial Standard (1983). *Kapi* (2 g) was mixed with 10 ml of distilled water. Then, the mixture was homogenized at a speed of 9500 rpm for 2 min using an IKA Labortechnik homogenizer (Selangor, Malaysia). Ten ml of formalin solution (38%, v/v; pH 9) was added and mixed well. The mixture was titrated with 0.1 N NaOH to obtain pH of 9.0. Formal nitrogen content was calculated and expressed as mg N/g sample using the following equation:

$$\text{Formal nitrogen content (mg N/g)} = \frac{\text{ml of NaOH (pH 7 - pH 9)} \times 0.1 \times 14}{\text{Weight of sample (g)}}$$

Ammonia nitrogen content was determined by the titration method as described by Thai Industrial Standard (1983). *Kapi* (2 g) was placed in 400 ml Kjeldahl flask containing 100 ml of distilled water and 3 g of MgO. The mixture was distilled and the distillate was collected in 50 ml of 4% (w/v) boric acid containing the mixed indicators (0.125 g methyl red and 0.082 g bromocresol green in 95% alcohol (100 ml): 0.1% methylene blue in distilled water with ratio of 5:1). The solution was then titrated

with 0.05 N H_2SO_4 to reach the end-point. Ammonia nitrogen content was calculated as follows:

$$\text{Ammonia nitrogen content (mg N/g)} = \frac{5.6 \times 0.05 \times \text{ml of } H_2SO_4}{\text{Weight of sample (g)}}$$

Amino nitrogen content was calculated based on the difference between formal and ammonia nitrogen contents (Thai Industrial Standard, 1983) and expressed as mg N/g sample.

7.4.3.3 Degree of hydrolysis (DH)

Firstly, the sample (1 g) was mixed with 9 ml of 5% (w/v) SDS. The mixture was homogenized at a speed of 11,000 rpm for 1 min. The homogenate was heated at 85°C for 30 min. The mixture was then subjected to centrifugation at 10,000×*g* for 15 min at room temperature (Model RC-B Plus centrifuge Newtown, CT, USA). Thereafter, 2.0 ml of 0.2 M phosphate buffer (pH 8.2) and 1.0 ml of 0.01% (w/v) TNBS solution was added. The solution was mixed thoroughly and placed in a temperature-controlled water bath (Memmert, Schwabach, Germany) at 50°C for 30 min in the dark. The reaction was terminated by adding 2.0 ml of 0.1 M sodium sulfite. The mixture was then cooled at room temperature for 15 min. The absorbance was read at 420 nm and free amino group content was expressed in terms of *L*-leucine. DH of *Kapi* was determined according to the method of Benjakul and Morrissey (1997) and expressed as degree of hydrolysis (% DH).

7.4.3.4 Peroxide values (PV)

PV was measured following the method of Maqsood and Benjakul (2010). Ground sample (1 g) was homogenized at a speed of 13,500 rpm for 2 min in 11 ml of chloroform/methanol (2:1, v/v). Homogenate was then filtered using a Whatman No. 1 filter paper. Two milliliters of 0.5% NaCl was then added to 7 ml of the filtrate. The mixture was vortexed at a moderate speed for 30 s and then centrifuged at 3000×*g* for 3 min to separate the sample into two phases. Two milliliters of cold chloroform/methanol (2:1) were added to 3 ml of the lower phase. Twenty-five

microliters of ammonium thiocyanate and 25 µl of iron (II) chloride were added to the mixture. Reaction mixture was allowed to stand for 20 min at room temperature prior to reading the absorbance at 500 nm. A standard curve was prepared using cumene hydroperoxide at the concentration range of 0.5-2 ppm. PV was reported as mg cumene preoxide/kg sample.

7.4.3.5 Thiobarbituric acid reactive substances (TBARS)

TBARS value was determined as per the method of Pongsetkul *et al.* (2015a). Ground sample (1 g) was mixed with 9 ml of 0.25 N HCl solution containing 0.375% TBA and 15% TCA. The mixture was heated in boiling water for 10 min, followed by cooling with the running water. The mixture was centrifuged at 4,000×*g* for 20 min (MIKRO20, Hettich Zentrifugan, Germany). The supernatant was collected and the absorbance was read at 532 nm using a UV-160 spectrophotometer (Shimadzu, Kyoto, Japan). A standard curve was prepared using malonaldehyde bis (dimethyl acetal) at concentrations ranging from 0 to 2 ppm. TBARS value was expressed as mg malonaldehyde (MDA)/kg sample.

7.4.3.6 Antioxidant activities

- Preparation of water extract

Water extract was prepared as per the method of Pongsetkul *et al.* (2015b). *Kapi* sample (1 g) was mixed with 25 ml of distilled water. The mixtures were homogenized at a speed of 11,000 rpm for 2 min, followed by centrifugation at 8,500×*g* for 15 min at room temperature. The supernatant was collected and adjusted to 25 ml using distilled water before analyses.

- Radical scavenging activities and reducing power

DPPH and ABTS radical scavenging activities as well as ferric reducing antioxidant power (FRAP) were determined according to the method of Faithong and Benjakul (2012). The activity was expressed as µmol Trolox equivalents (TE)/g sample. For the determination of DPPH radical scavenging activity, water extract of samples (1.5 ml) was added with 1.5 ml of 0.15 mM 2,2-diphenyl-1-picrylhydrazyl

(DPPH) in 95% ethanol. The mixture was then mixed vigorously and allowed to stand for 30 min in dark at room temperature. The resulting solution was measured at 517 nm using an UV-1601 spectrophotometer. The blank was prepared in the same manner except that distilled water was used instead of the sample. The standard curve was prepared using Trolox in the range of 10-60 μM.

To examine ABTS radical scavenging activity, the stock solutions including 7.4 mM ABTS solution and 2.6 mM potassium persulphate solution were prepared. The working solution was made by mixing two stock solutions in equal quantities and allowed them to react in the dark for 12 h at room temperature. The solution was then diluted by mixing 1 ml of ABTS solution with 50 ml of methanol to obtain an absorbance of 1.1 (±0.02) at 734 nm using an UV-1601 spectrophotometer. ABTS solution was prepared freshly for each assay. To initiate the reaction, 150 μl of sample were mixed with 2.85 ml of $ABTS^{\bullet+}$ solution. The mixture was incubated at room temperature for 2 h in dark. The absorbance was then read at 734 nm using an UV-1601 spectrophotometer. Distilled water was used instead of the sample and prepared in the same manner to obtain the blank. A Trolox standard curve (50-600 μM) was prepared.

For FRAP, the stock solutions included 10 mM TPTZ (2,4,6-tripyridyl-s-triazine) solution in 40 mM HCl, 20 mM $FeCl_3.6H_2O$ solution and 300 mM acetate buffer (pH 3.6). The working solution was prepared freshly by mixing 25 ml of acetate buffer, 2.5 ml of TPTZ solution and 2.5 ml of $FeCl_3.6H_2O$ solution. The mixture was incubated at 37°C for 30 min and was referred to as FRAP solution. The sample (150 μl) was mixed with 2.85 ml of FRAP solution. The mixture was allowed to stand in dark for 30 min at room temperature. Ferrous tripyridyltraizine complex, colored product, was measured by reading the absorbance at 593 nm. The blank was prepared in the same manner except that distilled water was used instead of the sample. The standard curve was prepared using Trolox ranging from 50 to 600 μM.

- Metal chelating activity

Metal chelating activity was assayed according to the method of Pongsetkul *et al.* (2015b). Sample (220 μl) was mixed with 5 μl of 2 mM $FeCl_2$ and 10

μl of 5 mM ferrozine. The mixture was allowed to stand at room temperature for 20 min. Absorbance at 562 nm was read. EDTA with the concentrations of 0-30 μM was used as standard. Metal chelating activity was expressed as μmole EDTA equivalent (EE)/g sample.

- Hydrogen peroxide and singlet oxygen scavenging activities

Hydrogen peroxide and singlet oxygen scavenging activities were investigated as described by Pongsetkul *et al.* (2015b). The activities were expressed as μmol Trolox equivalents (TE)/g sample.

To determine hydrogen peroxide scavenging activity, the extract (3.4 ml) was mixed with 600 μl of 43 mM hydrogen peroxide in 0.1 M phosphate buffer (pH 7.4). The absorbance at 230 nm of the reaction mixture was recorded after 40 min of reaction at 25°C. For the blank, hydrogen peroxide was omitted and replaced by 0.1 M phosphate buffer (pH 7.4). Trolox (0-10 mM) was used as standard.

For determination of singlet oxygen scavenging activity, the chemical solutions and the extract were prepared in 45 mM sodium phosphate buffer (pH 7.4). The reaction mixture consisted of 0.4 ml of extract, 0.5 ml of 200 μM *N,N*-dimethyl para-nitro-soaniline (DPN), 0.2 ml of 100 mM histidine, 0.2 ml of 100 mM sodium hypochlorite, and 0.2 ml of 100 mM H_2O_2. Thereafter, the total volume was made up to 2 ml with 45 mM sodium phosphate buffer (pH 7.4). The absorbance of the reaction mixture was measured at 440 nm after incubation at room temperature (25°C) for 40 min. Blank was run for each sample in the same manner, except DPN, histidine, and NaOCl solutions were replaced by sodium phosphate buffer. A standard curve of Trolox (0-10 mM) was prepared.

7.4.4 Statistical analysis

Completely randomized design (CRD) was used throughout the study. All experiments were run in triplicate. Data were subjected to analysis of variance (ANOVA), and mean comparisons were carried out by the Duncan's multiple range test. Independent T-test was performed for pair comparison (Steel *et al.,* 1980). Analysis were performed using SPSS statistic program (Version 10.0) (SPSS, 1.2,

1998). For PCA (Principal Component Analysis), the XLSTAT Software (XLSTAT, 2008, Addinsoft, New York, NY) was used.

7.5 Results and discussion

7.5.1 Autolysis of *A. vulgaris* and *M. lanchesteri*

7.5.1.1 Effect of temperature and pH

Autolytic degradation of *A. vulgaris* and *M. lanchesteri* as affected by temperature and pH is shown in Figure 20A and 20B, respectively. Maximal autolytic activities were found at 55 and 60°C for *A. vulgaris* and *M. lanchesteri,* respectively. At the physiological pH, autolytic activity increased markedly from 30°C and reached the maximum at 55 and 60°C for *A. vulgaris* and *M. lanchesteri,* respectively. With increasing temperatures, the activity was sharply decreased. At high temperature, unfolding or structural changes of proteases in both shrimps might occur, leading to a loss in proteolytic activity (Klomklao *et al.,* 2008). For the pH profile, the optimum pHs of autolysis, determined at the optimal temperature, were 7 for *A. vulgaris* and 8 for *M. lanchesteri.* The activity was drastically decreased when samples were subjected to very acidic or alkaline pHs. The increased repulsion between charged residues of protein molecules, associated with conformational changes, led to the denaturation of proteases under those conditions (Klomklao *et al.,* 2008). For both shrimp species, autolysis was most likely mediated by heat-activated proteases that were active at neutral or slightly alkaline pHs. Optimal temperature and pH for autolysis of both species tested were similar to those of proteases from Antarctic krill (*Euphausia superba*) (pH 8.2, 60°C) (Osnes and Mohr, 1985), North Pacific krill (*Euphausia pacifica*) (pH 8-9, 50°C) (Sun *et al.,* 2014), hepatopancreas of fresh water prawn (*Macrobrachium rosenbergii*) (pH 7, 60°C) (Sriket *et al.,* 2011) or Pacific brown shrimp (*Penaeus californiensis*) (pH 8, 50°C) (Vega-Villasante *et al.,* 1995), etc.

During *Kapi* production, salted shrimps were exposed to varying temperatures throughout the process from approximately 27 to 45°C (data not shown). Endogenous proteases of both *A. vulagris* and *M. lanchesteri* with the optimal temperature around 55-60°C were postulated to play a role in proteolysis at

aforementioned temperatures used for *Kapi* production. The differences in pH and temperature profiles of autolysis between both species might determine the degradation rate of proteins during *Kapi* process, in which the formation of degradation products could be varied. As a consequence, autolysis mediated by various proteases in both shrimps might have the impact on the characteristics of resulting *Kapi* differently.

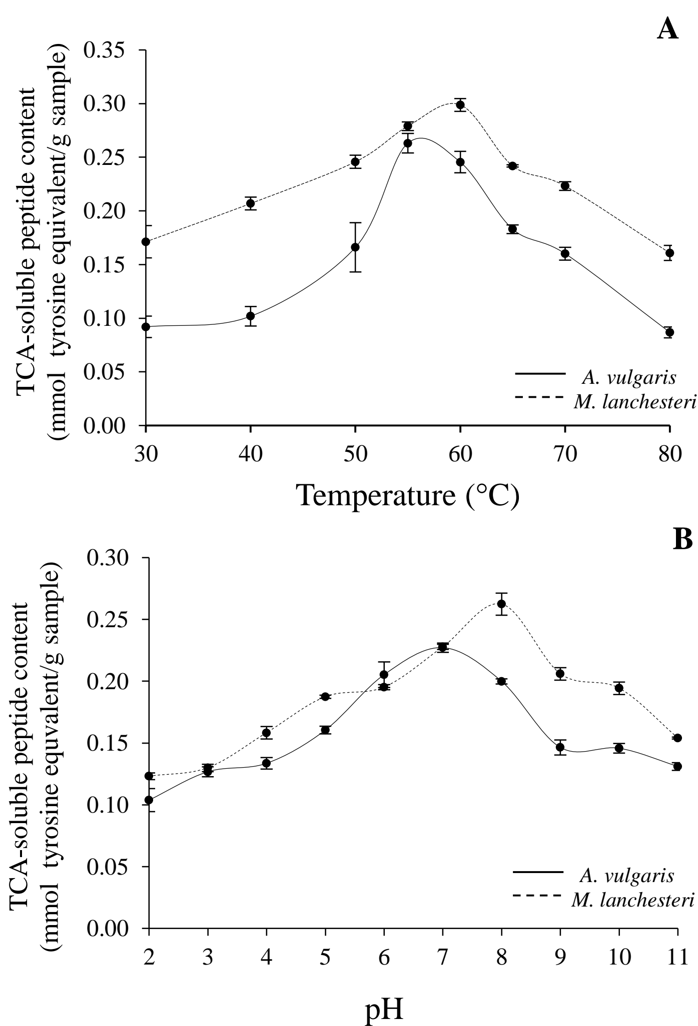

Figure 20. Temperature profile (A) and pH profile (B) of autolysis of *A. vulgaris* and *M. lanchesteri.* Autolytic activity was expressed as mmol tyrosine equivalent/g sample. Bars represent the standard deviation from triplicate determinations.

7.5.1.2 Effect of salt concentration

A sharp decrease in autolysis was observed as the concentration of salt increased up to 10% (Figure 21). The loss in activity more than 50% was noticeable when the concentration of NaCl was above 20% (w/v) in both shrimps. The losses in activity occurred as NaCl concentration increased, probably owing to the partial denaturation of proteases caused by the "salting out" effect (Klomklao *et al.,* 2008). The result revealed that NaCl at high concentration (15-25%), which is commonly used for *Kapi* production, could lower autolysis mediated by endogenous proteases in raw material. When NaCl concentration was above 20%, more activity of *M. lanchesteri* sample was retained, compared with *A. vulgaris* sample. The result suggested that the autolysis of *M. lanchesteri* in the presence of high salt took place to a higher degree than that of *A. vulgaris.* Varying degradation of proteins during *Kapi* fermentation, influenced by different raw material and concentration of salt, could led to the different characteristics of final product (Pongsetkul *et al.,* 2015a).

7.5.1.3 Effect of various protease inhibitors

Various protease inhibitors exhibited the inhibition toward autolysis of *A. vulgaris* and *M. lanchesteri* differently as shown in Figure 22. For autolysis of *A. vulgaris* at pH 7, PMSF and SBTI, the specific inhibitors for serine proteases, showed the highest inhibitory activity (81.63% and 59.46%, respectively). It was noted that TLCK and TPCK, specific inhibitor of trypsin and chymotrypsin, respectively, also showed high inhibitory activity. Furthermore, autolytic activity of *A. vulgaris* was slightly inhibited (less than 25%) by other inhibitors, including aspartic acid protease inhibitor (pepstatin A), metallo-protease inhibitor (EDTA) and cysteine protease inhibitor (E-64, iodoacetic acid). Thus, there were several kinds of proteases present in *A. vulgaris*. Similar inhibition of autolysis in *M. lanchesteri* was observed with those inhibitors. All serine protease inhibitors (SBTI, PMSF, TLCK and TPCK) had high inhibitory effect, compared with others. The result was in agreement with our previous study, in which both shrimps, *A. vulgaris* and *M. lanchesteri,* contained several groups of proteases, but serine proteases were dominant, particularly trypsin or chymotrypsin-like enzymes (Pongsetkul *et al.,* 2016b). Several crustaceans including *Penaeus*

vannamei (Garcia-Carreno *et al.,* 2008), *Macrobrachium rosenbergii* (Sriket *et al.,* 2012) and *Penaeus californiensis* (Navarrete-del-Toro *et al.,* 2015), etc. contained serine proteases as the major enzymes. The result indicated that digestive tract or organs in whole shrimp used for *Kapi* production contributed to the proteolysis or autolysis of shrimp during *Kapi* processing.

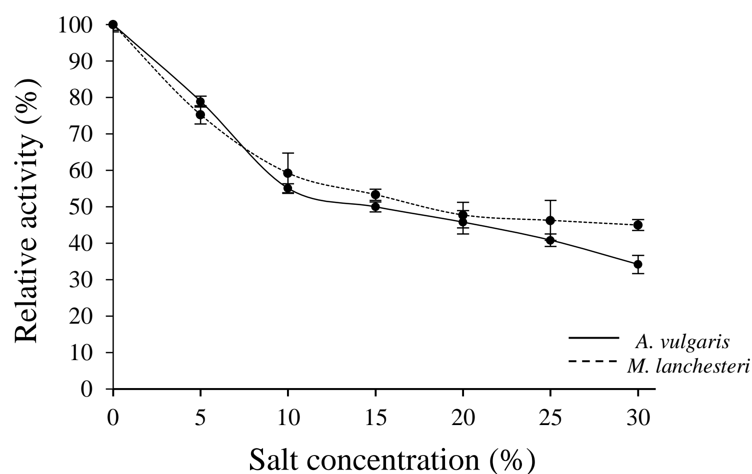

Figure 21. Effect of salt concentration on autolysis of *A. vulgaris* and *M. lanchesteri.* Autolytic activity was expressed as mmol tyrosine equivalent/g sample.

Bars represent the standard deviation from triplicate determinations.

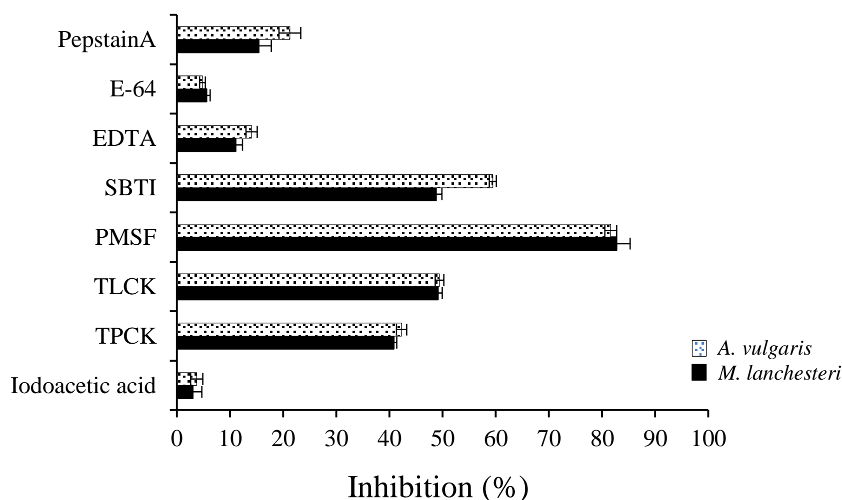

Figure 22. Effect of various inhibitors on autolysis of *A. vulgaris* and *M. lanchesteri.* Autolytic activity was expressed as mmol tyrosine equivalent/g sample.

Bars represent the standard deviation from triplicate determinations.

7.5.2 Properties of resulting *Kapi*

7.5.2.1 Formal, ammonia and amino nitrogen contents

Formal, ammonia and amino nitrogen contents of KA and KM are shown in Table 22. KA had the higher formal nitrogen content (14.92 mg N/g sample), compared with KM (11.76 mg N/g sample) ($p < 0.05$), suggesting the greater cleavage of peptides caused by endogenous or microbial proteases in the former. Generally, the formal nitrogen content has been used to indicate the degree of protein hydrolysis (Klomklao *et al.,* 2006). There was no difference in ammonia nitrogen content between these two samples ($p > 0.05$). The ammonia nitrogen content indicates the breakdown of soluble protein and peptides into free amino acid and volatile nitrogen (Pongsetkul *et al.,* 2014). The volatile nitrogenous compounds might contribute to different characteristics of the products, especially flavor. Amino nitrogen content, calculated based on the differences between formal nitrogen content and ammonia nitrogen content, represents the amount of primary amino group of the sample (Klomklao *et al.,* 2006). KA had the higher amino nitrogen content ($p < 0.05$). The result reconfirmed that higher cleavage of peptides was obtained in KA. Therefore, different shrimps determined the rate of degradation, more likely associated with different autolytic activities.

Table 22. Nitrogen contents, DH and lipid oxidation products of *Kapi* produced from *A. vulgaris* and *M. lanchesteri*

Compositions/Characteristics	KA	KM
Nitrogen content (mg N/g sample)		
Formal nitrogen content	14.92±1.20a	11.76±0.13b
Ammonia nitrogen content	1.10±0.19a	1.03±0.02a
Amino nitrogen content	13.82±1.03a	10.72±0.13b
Degree of hydrolysis (%)	15.93±0.51a	13.23±0.46b
Lipid oxidation products		
PV (mg cumene/ kg sample)	0.20±0.23a	0.18±0.251a
TBAR (mg MDA/ kg sample)	0.55±0.16a	0.28±0.30b

* KA, KM: *Kapi* produced from *A. vulgaris* and *M. lanchesteri*, respectively.
Values are given as mean ± SD (n = 3). Different lowercase superscripts in the same row indicate the significant difference ($p < 0.05$).

7.5.2.2 Degree of Hydrolysis

Degree of hydrolysis (DH) is the measure of the extent of cleavage of peptide linkages. DH close to 100% means that all proteins in the sample are completely hydrolyzed to free amino acids (Panyam and Kilara, 1996). As shown in Table 22, DH of KA and KM was 15.93 and 13.23%, respectively. The higher DH of KA correlated well with the higher degradation of proteins as indicated by the higher formal nitrogen content and amino nitrogen content. The result suggested that *A. vulgaris* was more pronounced in protein degradation during *Kapi* fermentation, compared with *M. lanchesteri*. Degradation of proteins in shrimp resulted in an increase in peptides and free amino acids and could be related with the development of flavor and odor of *Kapi*. Compounds such as peptides and amino acids contribute to the taste or flavor in a complex manner in the resulting *Kapi* (Visessanguan *et al.,* 2004).

7.5.2.3 Lipid oxidation products

Lipid oxidation of KA and KM was monitored by measuring PV and TBARS values, representing the primary and secondary oxidation products, respectively (Table 22). PV of KA and KM were 0.20 and 0.18 mg cumene/kg sample, respectively, suggesting that lipid oxidation occurred during processing and fermentation of *Kapi* to some extent. There was no difference in PV between these two samples ($p > 0.05$). However, KA had the higher TBARS value (0.55 mg MDA/kg sample), compared with KM (0.28 mg MDA/kg sample) ($p < 0.05$). The higher TBARS value of KA sample might probably due to the greater decomposition of hydroperoxides into the secondary oxidation products, especially aldehydes, in the later stage of lipid oxidation. Furthermore, during fermentation, autolysis or endogenous enzymatic activity might cause the disruption of the organelles associated with the release of pro-oxidants as well as reactants (Takeungwongtrakul and Benjakul, 2013). This resulted in the enhanced lipid oxidation in *Kapi.* Lipid oxidation is one of the deteriorative reactions causing the unacceptability of fish and shrimp product (Nirmal and Benjakul, 2009). Nevertheless, some lipid oxidation products in both KA and KM might contribute to typical odor and flavor of *Kapi*, leading to differences in acceptability of resulting *Kapi* produced from different shrimps.

7.5.2.4 Antioxidative activities

Water extracts of both KA and KM showed antioxidative activities differently as shown in Figure 23. Water extract of KM showed the higher DPPH radical scavenging activity (8.82 μmol TE/g sample) than that of KA (8.20 μmol TE/g sample), indicating that peptides or free amino acids in KM had the higher ability in donating hydrogen atom to free radicals, in which the propagation process of oxidation could be retarded (Peralta *et al.,* 2008). There was no difference in ABTS radical scavenging between KA and KM ($p > 0.05$). In general, ABTS assay is an excellent tool for determining both hydrophilic and lipophilic antioxidants for hydrogen donating (scavengers of aqueous phase radicals) and chain breaking (scavenger of lipid peroxyl radicals) (Leong and Shui, 2002). Binsan *et al.* (2008) reported that antioxidants in shrimp paste were more likely water soluble peptides. Furthermore, other antioxidative compounds including MRPs were also present in salted shrimp paste. Those peptides or MRPs were mostly hydrophilic in nature and were extracted into water effectively. However, KA showed the higher antioxidative activities including FRAP, metal chelating activity, hydrogen peroxide scavenging activity and singlet oxygen scavenging activity, compared with KM ($p < 0.05$). FRAP is generally used to measure the capacity of a substance in reducing TPTZ-Fe(III) complex to TPTZ-Fe(II) complex (Faithong and Benjakul, 2012). The higher FRAP activity in KA suggested the higher capability of providing the electron. Metal chelating activity of water extract of KA and KM were 3.21 and 2.93 μmol EE/g sample, respectively. The higher metal chelating activity of water extract of KA demonstrated the higher capacity of iron binding. The extracts could reduce the redox potential, thereby stabilizing the oxidized form of the metal ion (Oktay *et al.,* 2003). Moreover, the higher capacity of scavenging of hydrogen peroxide (H_2O_2) and singlet oxygen ($^1O_2$) of water extracts was also found in KA sample ($p < 0.05$). H_2O_2 is a reactive non radical, which can permeate biological membranes and is concerted to more reactive species such as hydroxyl radical and singlet oxygen (Choe and Min, 2005). It is the precursor for the generation of hydroxyl radical, which is a strong initiator of lipid oxidation (Suh *et al.,* 2011). Thus, the removal of H_2O_2 is very important for antioxidant defence in cell or food systems. KA

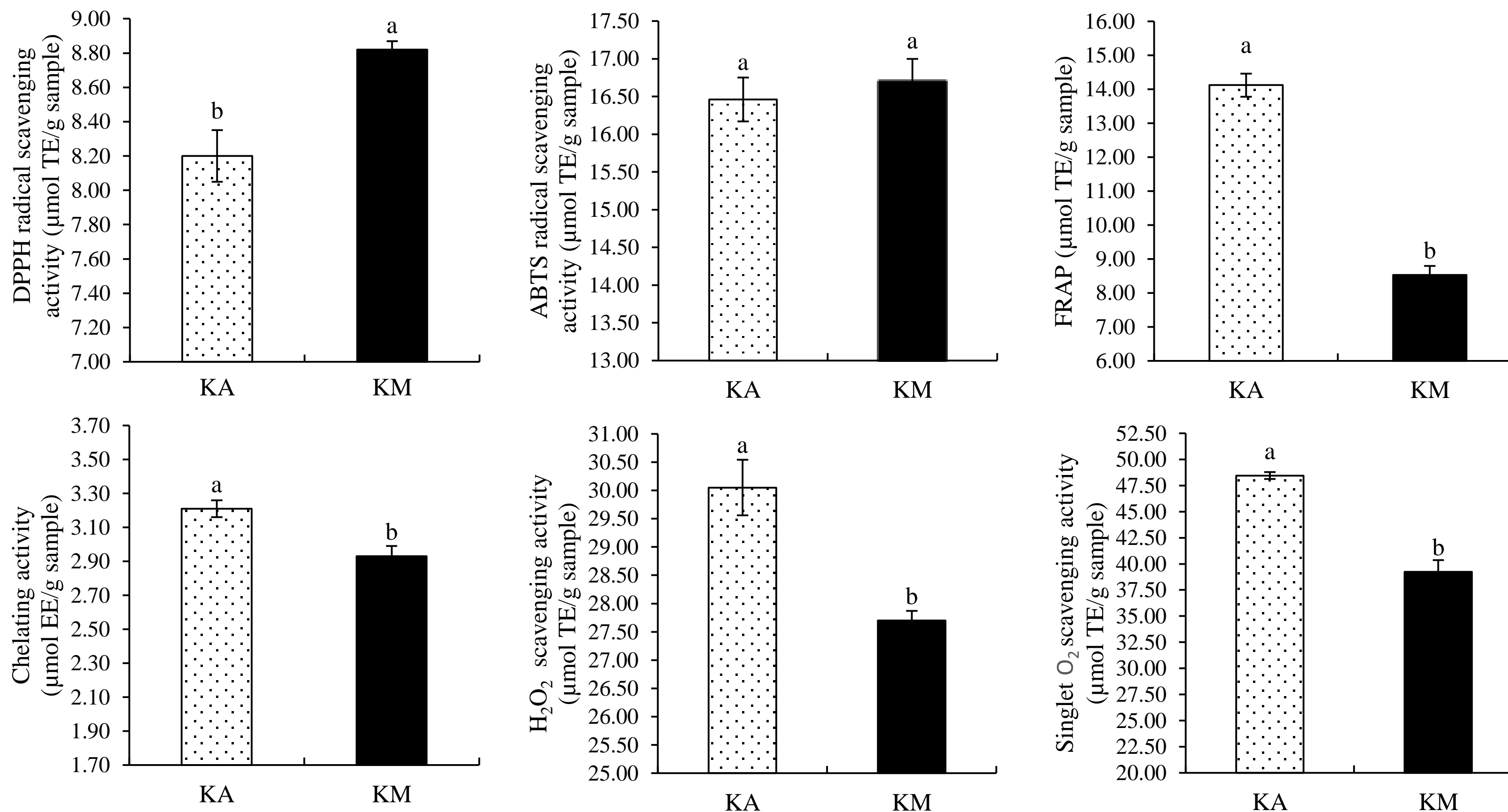

Figure 23. Antioxidative activities of water extract from *Kapi* produced from *A. vulgaris* (KA) and *M. lanchesteri* (KM).

Bars represent the standard deviation from triplicate determinations. Different lowercase letters on the bars indicate the significant difference ($p < 0.05$).

exhibited higher ability for scavenging H_2O_2 than KM ($p < 0.05$). This might be governed by the differences in peptides between both *Kapi* samples. Gou *et al.* (2009) reported that dipeptide containing tyrosine residue at its C-terminus was associated with strong hydrogen peroxide scavenging activity. The higher singlet oxygen scavenging activity was also observed in KA sample ($p < 0.05$). Singlet oxygen, which is a highly reactive, electrophilic and non-radical molecule, can be formed by the reaction between photosensitizers and triplet oxygen in the presence of light (Min and Boff, 2002). Singlet oxygen can directly react with electron-rich double bonds of unsaturated fatty acids without the formation of free-radical intermediates (Choe and Min, 2005).

Overall, both KA and KM might contain various antioxidant peptides with different modes of action. The different raw material might be degraded to varying extents, in which peptides could be produced in different fashions. Pongsetkul *et al.* (2015b) found that different commercial salted shrimp pastes obtained from different parts of Thailand had varying antioxidative activities.

7.6 Conclusion

A. vulgaris and *M. lanchesteri* contained serine proteases as the dominant enzymes with optimum pH and temperature of 7, 55°C and 8, 60°C, respectively. Both shrimps could be used as alternative raw material for *Kapi* production because of their high availability throughout the years and lower price, compared with krill (*Mesopodopsis orientalis*), typically used as the raw material. However, the differences in some properties were obtained. *Kapi* produced from *A. vulgaris* generally showed higher protein degradation, lipid oxidation and also possessed the higher antioxidative activities, compared with *Kapi* produced from *M. lanchesteri.* Therefore, different shrimp with different endogenous proteases associated with varying autolysis affected the characteristics and antioxidative properties of *Kapi.*

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CHAPTER 8

CHEMICAL COMPOSITIONS, VOLATILE COMPOUNDS AND SENSORY PROPERTY OF SALTED SHRIMP PASTE (*KAPI*) PRODUCED FROM *ACETES VULGARIS* AND *MACROBRACHIUM LANCHESTERI*

8.1 Abstract

Chemical compositions, volatile compounds and sensory property of *Kapi* produced from two shrimp species, *Acetes vulgaris* and *Macrobrachium lanchesteri,* were comparatively determined. Relationships between the volatiles and sensory scores of the samples, compared with commercial *Kapi* samples, were also investigated using principal component analysis (PCA). *Kapi* produced from *M. lanchesteri* (KM) had the higher protein content but lower fat content, compared with that from *A. vulgaris* (KA) ($p < 0.05$). However, KA showed higher browning intensity (A_{420}) and fluorescence intensity than KM, indicating browner and more yellowish color of the former. Both KA and KM contained varying volatile compounds, but *N*-containing compounds were predominant. Based on PCA of KA, KM and commercial *Kapi*, the intensity of *N*-containing compounds correlated well with sensory property. KA generally contained higher *N*-containing compounds and had higher flavor and overall likeness scores, compared with KM. Thus, *A. vulgaris* could serve as a potential alternative raw material for *Kapi* production.

8.2 Introduction

Salted or fermented krill or shrimp pastes are widely consumed in many Asian countries including Thailand (*Kapi*), Indonesia (*Terasi Udang*), Malaysia (*Belacan*), Philippines (*Bagoong-alamang*) or Vietnam (*Mam ruoc*), etc (Hajeb and Jinab, 2015). It is often used to enhance palatability of many foods by providing desirable flavor and salty or umami taste. In general, raw material, shrimp or krill/salt ratio, fermentation process and time can be varied, depending on regions or countries.

The different characteristics and properties of those products were reported (Peralta *et al.,* 2008).

Kapi, traditional salted shrimp paste of Thailand, is traditionally made from planktonous krill (*Mesopodopsis orientalis*). Since the last decade, krill stocks have drastically dropped by 3% per year (Meland and Willassen, 2007). Two species of small shrimps: *Acetes vulgaris* and *Macrobrachium lanchesteri* became potentially alternative source for *Kapi* production, because their high availability throughout the years, especially the southern part of Thailand. To produce salted shrimp paste, shrimp are mixed with salt and ground into a fine paste. Then, salted shrimps are sun-dried to reduce its moisture content, followed by fermentation at room temperature for approximately for 1 month (Pongsetkul *et al.,* 2014). During fermentation, the protein hydrolysis occurs and is mediated by the action of indigenous and microbial proteases. These phenomenon yields short chain peptides and free amino acids, which enhance the flavor and taste of final product (Pongsetkul *et al.,* 2015). Kim *et al.* (2014) reported that short chain peptides and free amino acids of Korean shrimp paste significantly increased during the fermentation period and could be responsible for the unique flavor of the product. The formation of Maillard reaction products (MRPs) were also observed throughout fermentation of Philippine salt-fermented shrimp paste and related with the darker/browner color of the final product (Peralta *et al.,* 2008). Moreover, some fermented shrimp products exhibited the strong antioxidant activities (Faithong *et al.,* 2010; Kleekayai *et al.,* 2015).

Flavor or aroma is one of the most important factors in *Kapi* quality (Phithakpol, 1993). The characteristic flavor and aroma are primarily due to protein and lipid degradation by autolytic and bacterial enzymes during fermentation, governed by different raw material, process employed, as well as strains of microorganism involved (Saisithi *et al.,* 1966). Several volatile components of shrimp paste products were associated with their flavors (Cha and Cadwallader, 1995; Pongsetkul *et al.,* 2014; Wittanalai *et al.,* 2011; Kang and Baek, 2014). Nevertheless, a little information regarding chemical compositions, especially volatiles as well as sensory property of *Kapi*, produced from *A. vulgaris* and *M. lanchesteri* has been reported.

8.3 Objective

To comparatively characterize some chemical compositions, volatile compounds and sensory property of *Kapi* produced from both shrimps, *A. vulgaris* and *M. lanchesteri*. To study the relationship between volatile compounds and sensory properties of both *Kapi,* compated with commercial *Kapi* by using principal component analysis (PCA).

8.4 Materials and methods

8.4.1 Sample collection

Shrimp *A. vulgaris* (average body length 15.6±1.4 mm, average wet weight 0.0413±0.0098 g, n=20) and *M. lanchesteri* (average body length 27.5±1.9 mm, average wet weight 0.0701±0.0107 g, n=20) were caught from the coast in Ko-yo and The-Pha in Songkhla province, Thailand, respectively. After capture, shrimp were transported in ice with a shrimp/ice ratio of 1:2 (w/w) in a polystyrene container to the Department of Food Technology, Prince of Songkla University, Hat Yai, Thailand, within approximately 2 h.

8.4.2 Preparation of *Kapi*

Shrimps were mixed with salt at the ratio of 5:1 (w/w) and transferred into the basket, covered with the cheese cloth. The mixture was kept at room temperature (28-32°C) overnight. Then, the drained sample were mashed or pounded thoroughly and spread out on fiberglass mats to dry with sunlight. The drying step was continued until samples disintegrate and turned from pink to dark purplish brown (with the moisture content of 35-40%). Subsequently, samples were transferred into earthen jars, covered with plastic bag tightly (close system), and allowed to ferment at room temperature. After 30 days of fermentation, *Kapi* were collected and referred to as KA (*Kapi* produced from *A. vulgaris*) and KM (*Kapi* produced from *M. lanchesteri*). The obtained samples were subjected to analyses.

8.4.3 Characterization of *Kapi* produced from *A. vulgaris* and *M. lanchesteri*

8.4.3.1 pH and water activity (A_w)

The pH of samples was measured according to the method of Nirmal and Benjakul (2009) using a pH meter (Sartorius, Gottingen, Germany). A_w of *Kapi* was determined using a water activity analyzer (Thermoconstanter, Novasina, Switzerland).

8.4.3.2 Proximate composition and salt content

Moisture, ash, fat, protein and carbohydrate contents of *Kapi* were determined according to AOAC method (2000) with the analytical No. of 35.1.13, 35.1.14, 35.1.25, 35.1.15 and 35.1.16, respectively. Salt content was determined with the analytical number of 35.1.18 and was expressed as %NaCl (AOAC, 2000).

8.4.3.3 Color

Color of samples was determined using a colorimeter (ColourFlex, Hunter Lab Reston, VA) with the CIE system. L^* (lightness), a^* (redness/greenness), b^* (yellowness/blueness), ΔE^* (total difference of color) and ΔC^* (the difference in chroma) were recorded as described by Pongsetkul *et al.* (2014).

8.4.3.4 Browning products

- Preparation of water extract

Kapi (1 g) was mixed with 25 ml of distilled water. The mixtures were homogenized at a speed of 11,000 rpm for 2 min, followed by centrifugation at 8,500×*g*

for 15 min at room temperature. The supernatant was collected and adjusted to 25 ml using distilled water before analyses.

- Measurement of browning products

After being diluted, the water extracts were measured for browning intensity (A_{420}) and Maillard reaction products (A_{280} and A_{295}) using the UV-1601 spectrometer (Shimadzu, Kyoto, Japan). The fluorescence intensity at an excitation wavelength of 347 nm and emission wavelength of 415 nm was also determined using a fluorescence spectrophotometer RF-1501 (Shimadzu, Kyoto, Japan).

8.4.3.5 Volatile compounds

Kapi produced from *A. vulgaris* and *M. lanchesteri*, as well as three commercial *Kapi* produced from krill (*Mesopodopsis orientalis*) obtained from different provinces in Thailand, including Krabi, Samut Sakhon and Rayong were characterized on their volatile compounds using a solid-phase microextraction gas chromatography mass spectrometry (SPME GC-MS). To extract volatile compounds, samples (5 g) were mixed with 10 ml of deionized water. The mixture was homogenized at a speed of 13,000×*g* for 1 min to disperse the sample. The homogenate was placed in a 20-ml headspace vial (Supelco, Bellefonte, PA, USA) and determined as per the method of Iglesias and Medina (2008) as detailed by Takeungwongtrakul and Benjakul (2013). Volatile compounds were identified and expressed in the terms of relative abundance.

8.4.3.6 Sensory evaluation

Kapi produced from *A. vulgaris* and *M. lanchesteri*, as well as commercial *Kapi* produced from krill (*Mesopodopsis orientalis*) obtained from different provinces in Thailand, including Krabi, Samut Sakhon and Rayong were subjected to evaluate on sensory score. The 50 untrained panelists, who consumed *Kapi* regularly, were used for evaluation. The samples were wrapped with aluminium foil and heated in hot air oven at 60°C for 30 min. After cutting into small pieces (2×2×1 cm^2), samples were placed in 15-ml plastic cup, covered with lids and left at room

temperature for 30 min before serving. The panelists were asked to open the lid and sniff. Between the samples, panelists rinsed their mouth with water, cucumber or cracker. Scores for appearance, color, odor, flavor, texture and overall likeness using a 9-point hedonic scale were recorded.

8.4.4 Principal component analysis (PCA)

PCA was performed to access the relationship between volatile compounds, odor-liking, flavor-liking and overall-liking score of *Kapi* produced from *A. vulgaris* and *M. lanchesteri*, as well as commercial *Kapi* produced from krill (*Mesopodopsis orientalis*) obtained from different provinces in Thailand, including Krabi, Samut Sakhon and Rayong.

8.4.5 Statistical analysis

Completely randomized design (CRD) was used throughout the study. All experiments were run in triplicate. Data were subjected to analysis of variance (ANOVA), and mean comparisons were carried out by the Duncan's multiple range test. Independent T-test was performed for pair comparison (Steel *et al.,* 1980). Analysis were performed using SPSS statistic program (Version 10.0) (SPSS, 1.2, 1998). For PCA (Principal Component Analysis), the XLSTAT Software (XLSTAT, 2008, Addinsoft, New York, NY) was used.

8.5 Results and discussion

8.5.1 Characteristics and properties of *Kapi*

8.5.1.1 pH, A_w and proximate composition

As shown in Table 23, *Kapi* produced from *A. vulgaris* (KA) and *M. lanchesteri* (KM) had the neutral pH. KM had the slightly higher basic pH (7.27), compared with KA (7.16) ($p < 0.05$). The slightly basic pH might be caused by the basic degradation products generated during postmortem storage of raw material or the formation of volatile base compounds such as ammonia during fermentation of samples (Pongsetkul *et al.,* 2014). The pH of Korean dried shrimp paste was in the range of

6.83-7.23 (Cho and Kim, 2010), while Fillipino fermented shrimp paste had pH of 7.50 (Montano *et al.,* 2001). The pHs of those shrimp pastes were similar to those of *Kapi* in the present study. Both samples had no differences in water activity (A_w) ($p > 0.05$). A_w of both *Kapi* was in the range of 0.6-0.7, which could be classified as an intermediate moisture food (Fennema, 1996). This was associated with the prolonged shelf-life of this product due to the lowered growth of food pathogens and spoilage microorganisms. Low A_w of *Kapi* samples was in agreement with the low moisture content. There was no difference in moisture content between KA (33.93%) and KM (34.28%) ($p > 0.05$). No differences in carbohydrate, ash and salt contents were found between KA and KM ($p > 0.05$). KM had the higher protein content (28.48%), compared with KA (26.20%) ($p < 0.05$). Conversely, KA had the higher fat content (3.91%) than KM (2.36%) ($p < 0.05$). KA and KM had a high salt content (22.45-22.88%), related with their low A_w. The large amount of salt added, as well as the presence of inorganic substances in the shell of shrimp used as raw material, resulted in the high ash content in both samples (33.13-32.94%). It could be inferred that different shrimps yielded *Kapi* with different compositions.

8.5.1.2 Color

KA and KM had different color characteristics as depicted in Table 23. KA showed the lower L^*-value but higher b^*, ΔC^* and ΔE^*-value ($p < 0.05$). However, no difference in a^*-value between both samples was observed ($p > 0.05$). The result suggested that KA showed browner and more yellowish color than KM. Differences in color of both samples might be due to the different amount and type of pigments in raw material (*A. vulgaris* and *M. lanchesteri*). In general, carotenoids, especially astaxanthin, provide the desirable reddish-orange color in crustaceans (Higuera-Ciapara *et al.,* 2006). During fermentation, free amino acids and small peptides could undergo Maillard reaction, thereby contributing to the brown color development (Lopetcharat *et al.,* 2001). Lipid oxidation was also associated with browning mediated by Maillard reaction (Yarnpakdee *et al.,* 2014). The carbonyl groups of aldehydes and ketone, the oxidation products, could react with amino groups of free amino acids or peptides generated during hydrolysis, leading to yellow or brown color development (Yarnpakdee *et al.,* 2014).

Table 23. Chemical compositions and characteristics of *Kapi* produced from *A. vulgaris* and *M. lanchesteri*

Compositions/Characteristics	KA*	KM
pH	7.16±0.01[b]	7.27±0.03[a]
Water activity (A_w)	0.662±0.00[a]	0.659±0.01[a]
Proximate composition		
Moisture	33.93±0.99[a]	34.28±0.83[a]
Crude protein	26.20±0.54[b]	28.48±0.63[a]
Crude fat	3.91±0.25[a]	2.36±0.87[b]
Ash	32.94±0.99[a]	33.13±0.12[a]
Carbohydrate	2.57±1.55[a]	1.16±0.69[a]
Salt	22.88±1.15[a]	22.45±1.65[a]
Colour		
L^*	40.92±0.32[b]	45.23±0.76[a]
a^*	9.53±0.52[a]	9.17±0.08[a]
b^*	18.11±0.14[a]	16.78±0.04[b]
ΔE^*	56.50±0.23[a]	52.13±0.69[b]
ΔC^*	19.55±0.16[a]	18.56±0.94[b]
Browning and Maillard reaction products		
A_{280}	0.90±0.09[a]	1.01±0.05[a]
A_{295}	0.83±0.02[a]	0.85±0.17[a]
Browning intensity (A_{420})	0.46±0.01[a]	0.32±0.02[b]
Fluorescence intensity	403.91±6.31[a]	315.88±6.01[b]

* KA, KM: *Kapi* produced from *A. vulgaris* and *M. lanchesteri*, respectively.
Values are given as mean ± SD (n = 3). Different lowercase superscripts in the same row indicate the significant difference ($p < 0.05$).

8.5.1.3 Browning and Maillard reaction products

Non-fluorescent and fluorescent intermediates of Maillard reaction products as well as browning intensity of both water extracts of KA and KM are presented in Table 23. A_{280} and A_{295} have been used to determine the formation of non-fluorescent intermediate compounds of the Maillard reaction (Binsan *et al.,* 2008). There were no differences in A_{280} and A_{295} between both samples ($p > 0.05$). However, the differences in fluorescent intermediate products was observed. KA had the higher fluorescence intensity (403.91), compared with KM (315.88) ($p < 0.05$). The result was in accordance with the higher browning intensity (A_{420}) found in KA. The relationship between browning intensity and fluorescence intensity suggested that a large proportion of fluorescent intermediate products was converted into brown polymers. Jing and Kitts (2002) reported that the development of fluorescent compounds occurred in the

Maillard reaction prior to the generation of brown pigments. Generally, both non-fluorescent and fluorescent intermediates are formed and turn into brown pigments in the Maillard reaction (Binsan *et al.,* 2008). Benjakul *et al.* (2005) revealed that the fluorescent intermediate was more reactive in formation of brown color than non-fluorescent compounds. The higher browning intensity of KA sample was in agreement with the browner color of this sample (Table 23). Thus, the differences in browning could affect the color and acceptability of *Kapi* to some degrees.

8.5.1.4 Volatile compounds

Fourty-two volatile compounds of KA, KM and three commercial *Kapi* samples were detected (Table 24). These were classified into 6 main groups including aldehydes (5), ketones (8), alcohols (10), *N*-containing compounds (8), hydrocarbon (6) and others (6). For aldehydes, 3-methyl-butanal, pentanal, heptanal and benzaldehyde were found in all samples, while hexanal was not observed in KA. Among all samples, KC1 showed the highest intensity of aldehydes (8.47%), followed by KC3 and KC2, indicating that commercial *Kapi* had the higher amount of aldehydes, compared with KA and KM. The presence of aldehydes and ketones are related with lipid oxidation during fermentation (Pongsetkul *et al.,* 2015a). Eusebio *et al.* (2010) reported that krill (*M. orientalis*) contained 4.1-10.6% fat, while the fat contents of *A. vulgaris* and *M. lanchesteri* were 4.62 and 3.93% (dry weight basis) as reported by Pongsetkul *et al.* (2015a) and Pongsetkul *et al.* (2016a), respectively. Krill or shrimp oil was reported to be rich in polyunsaturated fatty acids, which were prone to oxidation (Takeungwongtrakul and Benjakul, 2013). Benzaldehyde was reported to have a pleasant almond, nutty and fruity aroma (Cha and Cadwallader, 1995). 3-methyl-butanal is characterized by a green and fruity flavor and is generated via Strecker degradation through Maillard reactions of isoleucine (Cha and Cadwallader, 1995). Strecker aldehydes are present and known to be potent odorants in many seafood products (Casaburi *et al.,* 2008).

Ketones found in all samples included 1-(2-aminophenyl)-ethanone, 2-hexanone and 2-heptanone. Park *et al.* (2014) revealed that 2-hexanone and 2-heptanone were produced by oxidation or pyrolysis of polyunsaturated fatty acids and were involved in a nasty smell in seafood. KM had the higher intensity of ketones

(9.85%), compared with KA (4.00%), but lower than all commercial *Kapi* samples (7.17-12.68%).

Table 24. Volatile compounds of *Kapi* produced from *A. vulgaris*, *M. lanchesteri* and three commercial *Kapi*

Volatile compounds	Peak area (Abundance) × 10^6				
	KA*	KM	KC1	KC2	KC3
3-methyl-butanal	41.73	36.65	45.65	55.35	65.31
Pentanal	22.02	45.55	26.22	83.54	14.28
Hexanal	ND	81.12	60.06	14.27	51.11
Heptanal	44.45	12.03	55.55	ND	45.45
Benzaldehyde	128.95	44.09	91.02	45.62	133.41
Total Aldehydes (%)	**4.30%**	**6.06%**	**8.47%**	**7.01%**	**7.06%**
1-phenyl-ethanone	24.43	51.92	209.05	ND	13.34
1,2-diphenyl-ethanone	ND	58.06	ND	66.97	ND
1-(2-aminophenyl)-ethanone	55.91	16.32	44.41	29.29	54.41
2-pentanone	18.08	ND	ND	ND	ND
2-hexanone	55.12	72.8	22.88	27.77	105.14
2-heptanone	67.18	65.43	18.84	115.65	105.99
6-methyl-5-hepten-2-one	ND	12.02	15.65	105.59	16.62
3-octanone	ND	79.99	105.99	ND	18.84
Total Ketones (%)	**4.00%**	**9.85%**	**12.68%**	**12.18%**	**7.17%**
Benzenemethanol	152.22	225.09	105.05	24.99	206.12
2-butyl-ethanol	78.12	22.45	ND	13.38	ND
2-methyl, 1- propanol	113.13	ND	ND	95.15	22.25
1-butanol	ND	17.71	103.32	98.45	12.25
2-butanol	28.26	ND	21.13	33.42	44.78
3-methyl-butanol	114.95	ND	104.22	95.11	232.26
1-pentanol	72.28	ND	88.43	22.92	82.22
1-penten-3-ol	105.32	77.62	ND	140.15	125.11
5-methoxy-1-pentanol	622.1	620.53	102.34	ND	14.22
2,4-dimethyl-3-pentanol	ND	71.4	ND	11.41	ND
Total Alcohols (%)	**23.31%**	**29.59%**	**15.95%**	**18.87%**	**16.86%**
Methyl-pyrazine	225.25	205.55	99.55	104.52	351.12
2-ehtyl-6-methyl-pyrazine	338.11	113.95	ND	ND	11.08
3-ethyl-5-methyl-pyrazine	198.11	113.95	214.55	258.29	137.11
2,3-diethyl, 5-methyl-pyrazine	26.62	ND	44.13	ND	11.34
2,5-dimethyl-pyrazine	634.88	85.39	211.35	142.72	330.11
2,6-dimethyl-pyrazine	310.42	197.32	142.77	105.92	299.76
3-ethyl-2,5-dimethyl-pyrazine	505.55	313.14	225.57	129.99	146.52
2-ethyl-3,5-dimethyl-pyrazine	408.22	118.23	213.99	198.14	555.11
Total *N*-containing Compounds (%)	**47.98%**	**31.71%**	**35.04%**	**33.15%**	**42.01%**

ND: non-detectable

* KA, KM: *Kapi* produced from *A. vulgaris* and *M. lanchesteri*, KC1, KC2, KC3: Commercial *Kapi* produced from Krill *Mesopodopsis orientalis* from Krabi, Samut Sakhon and Rayong, respectively.

Table 24. Volatile compounds of *Kapi* produced from *A. vulgaris*, *M. lanchesteri* and three commercial *Kapi* (Cont.)

Volatile compounds	Peak area (Abundance) × 10^6				
	KA*	KM	KC1	KC2	KC3
2,6,10,14-tetramethyl-pentadecane	98.15	19.75	ND	ND	ND
3-tetradocene	24.46	5.51	ND	43.35	ND
2,3-butanediene	13.22	ND	28.01	28.01	ND
2-undecane	9.11	55.46	ND	44.13	79/82
Hexadecane	ND	6.63	102.22	18.83	ND
Total Hydrocarbon (%)	**2.63%**	**2.41%**	**3.96%**	**4.74%**	**1.82%**
Propanoic acid	12.28	ND	ND	ND	118.18
Butanoic acid	49.52	39.61	22.28	105.55	105.16
Methyl-ester-butanoic acid	104.35	23.35	50.05	48.71	12.22
Pentatonic acid	ND	0.95	99.41	ND	13.38
Phenol	209.55	510.01	505.93	455.15	705.66
1H-Indole	605.55	199.34	108.11	72.28	145.55
Total Others (%)	**17.78%**	**21.37%**	**23.90%**	**24.05%**	**25.09%**

ND: non-detectable
* KA, KM: *Kapi* produced from *A. vulgaris* and *M. lanchesteri*, KC1, KC2, KC3: Commercial *Kapi* produced from Krill *Mesopodopsis orientalis* from Krabi, Samut Sakhon and Rayong, respectively.

Among 10 alcohols found in *Kapi*, only benzenemethanol was obtained in all samples. This compound gives the almond-like odor in seafood (Park *et al.,* 2014). Michihata *et al.* (2002) noted that normal and branched alcohol, especially butanol derivatives, might be formed by microbial fermentation or the degradation products from lipid oxidation. The higher amount of 5-methoxy-1-pentanol was obtained in KA and KM, compared with commercial samples. The type and abundance of individual alcohol found in *Kapi* seemed to vary with different raw material used for production. However, alcohols might not have a paramount impact on *Kapi* flavor because of their high flavor thresholds (Cha and Cadwallader, 1995).

All *Kapi* samples contained *N*-containing compounds as dominant volatiles. KA had the highest abundance (47.98%), followed by KC3 (42.01%) and KC1 (35.04%), respectively. Major pyrazine compounds found in all samples included methylpyrazine, 3-ethyl-5-methylpyrazine, 3-ethyl-2,5-dimethyl-pyrazine, etc. These compounds contributed to prawn, roasted, nutty and dried seafood like odors, which were the desirable odor in dried fermented food (Jaffres *et al.,* 2011). Rodriguez-Bernaldo *et al.* (2001) reported that pyrazine compounds were generated in the samples

dried using thermal conditions i.e. spray drying and tray drying. The drying step with sunlight during *Kapi* production more likely contributed to the formation of these compounds. Pyrazines was thermally generated via Maillard reaction through Strecker degradations from various nitrogen sources such as amino acids in heat processed foods (Rodriguez-Bernaldo *et al.,* 2001). Furthermore, the presence of pyrazine indicated that browning reaction mediated by Maillard reaction occurred in *Kapi* during fermentation. Pyrazine derivative compounds were the major volatiles found in many fermented products including *Ishiru* (Japanese fish sauce) (Michihata *et al.,* 2002), *Noucmam* (Vietnamese fish sauce) (Lopetcharat *et al.,* 2001), fermented dried shrimp (*Acetes chinensis*) (Lu *et al.,* 2011) as well as *Kapi* (Cha and Cadwallader, 1995; Pongsetkul *et al.,* 2015a). This compound might contribute to flavor, color as well as antioxidative activity of *Kapi* to some extent.

Low abundance of hydrocarbons (1.82-4.74%) was obtained in *Kapi*. Those included 2,6,10,14-tetramethyl-pentadecane, 3-tetradocene, 2,3-butanediene, etc. Latorre-Moratalla *et al.* (2011) noted that most hydrocarbons, alkanes and alkenes, are mainly formed from lipid auto-oxidation of fatty acids released from triglycerides. Additionally, some acids were found in some *Kapi* samples. Propanoic acid, which found in KA and KC3, mainly contribute to oily notes in foods (Chung *et al.,* 2005). Butanoic acid and methyl-ester-butanoic acid were noticeable in all samples. These acids are known to have cheesy notes including cheesy, sharp, rancid, sweaty, and pungent (Chung *et al.,* 2005). Additionally, phenol and 1H-indole were also obtained in all samples. Cha and Cadwallader (1995) reported that phenol give an undesirable aroma in seafood. Indole is the degradation product from tryptophan and has been used as the index for shrimp spoilage (Casaburi *et al.,* 2008). Based on volatile compounds, *Kapi* produced from different shrimps contained varying amount and type of volatile compounds. This might be associated with different flavors and acceptability of various *Kapi*.

8.5.1.5 Sensory evaluation

Likeness scores of KA, KM, as well as three commercial *Kapi* are shown in Table 25. Generally, KC3 had the highest likeness score for all sensory characteristics including appearance, color, odor, texture, flavor and overall-liking

score ($p < 0.05$). There were no differences in appearance-liking score between all samples ($p > 0.05$). The highest color-liking score was obtained for KC1 (7.30), while KM had the lowest color-liking score (6.57). Pongsetkul *et al.* (2015a) suggested that *Kapi* with browner or darker color was more desirable. Lower L^* but higher b^*-value of KA (Table 23) indicated higher intensity of color, especially more yellowish or browner, than KM. This led to the higher color-liking score of KA. Furthermore, the lowest odor and flavor-liking scores were found in KM ($p < 0.05$). In general, the differences in sensorial characteristics of fermented food could be influenced by raw material used, ingredients, fermentation process and conditions (Beriain *et al.,* 2000). Therefore, it was likely that differences in compositions as well as autolysis in raw material contributed to varying likeness scores of *Kapi*. In the present study, odor and flavor mainly affected the sensory quality (overall-liking) of this product. Based on overall-liking score, KA and KC3 had the highest overall-liking score, compared with others ($p < 0.05$). The result indicated that *A. vulgaris* seemed to have high potential to become an alternative raw material for *Kapi* production.

Table 25. Likeness score of *kapi* produced from *A. vulgaris*, *M. lanchesteri* and three commercial *Kapi*

Attributes	Samples				
	KA*	KM	KC1	KC2	KC3
Appearance	7.27 ± 1.07^{a}	7.23 ± 1.11^{a}	7.10 ± 0.96^{a}	7.25 ± 1.13^{a}	7.15 ± 1.05^{a}
Color	7.10 ± 1.03^{b}	6.57 ± 1.05^{c}	7.30 ± 1.22^{a}	7.03 ± 0.54^{b}	7.20 ± 0.98^{ab}
Odor	7.25 ± 1.02^{a}	6.95 ± 0.44^{b}	7.30 ± 0.55^{a}	7.25 ± 0.63^{a}	7.35 ± 1.01^{a}
Texture	7.10 ± 0.97^{a}	6.95 ± 0.44^{ab}	6.53 ± 2.05^{b}	6.95 ± 0.67^{ab}	7.10 ± 0.58^{a}
Flavor	7.30 ± 0.80^{a}	6.83 ± 1.05^{c}	7.03 ± 1.01^{b}	7.10 ± 1.12^{b}	7.40 ± 0.22^{a}
Overall	7.43 ± 0.22^{a}	6.85 ± 0.77^{c}	7.05 ± 0.87^{b}	7.10 ± 0.22^{b}	7.40 ± 0.53^{a}

* KA, KM: *Kapi* produced from *A. vulgaris* and *M. lanchesteri*, KC1, KC2, KC3: Commercial *Kapi* produced from Krill *Mesopodopsis orientalis* from Krabi, Samut Sakhon and Rayong, respectively. Values are given as mean ± SD (n = 3). Different lowercase superscripts within the same column indicate the significant differences ($p < 0.05$).

8.5.2 Principal component analysis (PCA)

Relationships between volatile compounds of different *Kapi* samples including KA, KM and commercial *Kapi* and sensory score (odor, flavor and overall-

liking score) were studied using PCA (Figure 24). The first two principal components could be described as 87.26% of the variations in the data set. It was noticed that the first principal component, which was the direction of the maximum explained variance (47.96%), demonstrated a useful separation between groups of volatiles. From the loadings, the samples placed to the right along PC1 (KA and KC3) were characterized by higher intensity of *N*-containing compound, associated with the higher odor, flavor as well as overall-liking score. In contrast, samples placed to the left along PC1 (KM, KC1 and KC2) were described as higher intensity of other groups of volatiles including aldehydes, ketones, etc. Moreover, PC2, which explains a lower variance percentage (39.30%), revealed that commercial *Kapi* contained the higher intensity of aldehydes, ketones as well as hydrocarbon, compared with KA and KM. The total separation of high amount of alcohols in KM was also observed. However, alcohols seemed to have less effect on sensorial scores. Based on PCA results, it was possible to confirm that flavor-liking score was closely correlated with overall-liking score of *Kapi*. The highest overall-liking score in KA and KC3 samples (Table 25) might be caused by higher intensity of *N*-containing compounds. This result confirmed that *A. vulgaris* showed higher potential as an alternative raw material for production of *Kapi*, in comparison with *M. lanchesteri.*

8.6 Conclusion

Kapi produced from *A. vulgaris* and *M. lanchesteri* had different chemical compositions, physical and sensory properties. *Kapi* produced from *A. vulgaris* with browner color showed higher fat content, but lower protein content, compared with *Kapi* produced from *M. lanchesteri.* The former had higher likeness score that the latter. Volatile compounds of both samples were also different. *N*-containing compounds, which was predominant volatiles in *Kapi*, played a profound role in likeness of this product. Thus, *Kapi* could be prepared from *A. vulgaris* with comparable sensory property to commercial products.

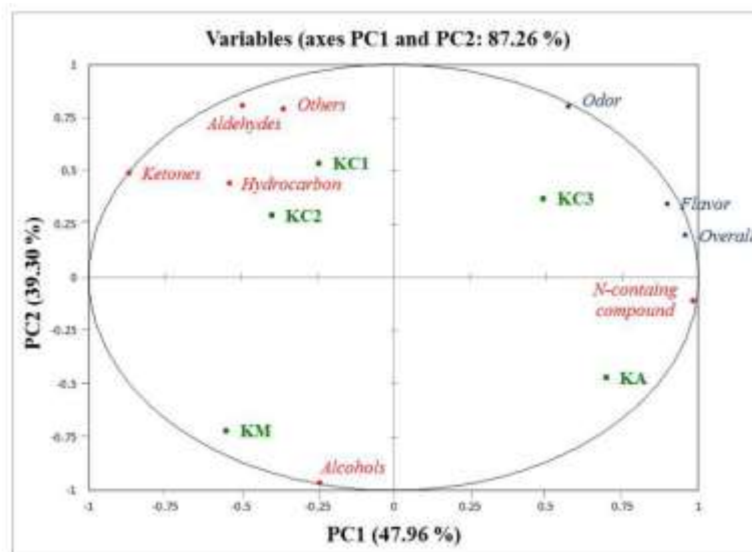

Figure 24. PCA score (samples, in bold, and groups of volatile compounds and sensorial characteristics, in italic) of *Kapi* produced from *A. vulgaris*, *M. lanchesteri* and three commercial *Kapi* samples.

*KA, KM: *Kapi* produced from *A. vulgaris* and *M. lanchesteri*, respectively. KC1, KC2, and KC3: Commercial *Kapi* produced from Krill *Mesopodopsis orientalis* from Krabi, Samut Sakhon and Rayong, respectively.

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CHAPTER 9

CHANGES IN VOLATILE COMPOUNDS, ATP-RELATED COMPOUNDS AND ANTIOXIDATIVE PROPERTIES OF *KAPI*, PRODUCED FROM *ACETES VULGARIS*, DURING PROCESSING AND FERMENTATION

9.1 Abstract

During salting, drying and fermentation for making *Kapi* (salted shrimp paste of Thailand), from *Acetes vulgaris*, Maillard reaction products (MRPs), adenosine triphosphate (ATP)-related compounds, antioxidative activities and volatile compounds were monitored. Varying colors were observed during processing. The results of the study showed that L^* and b^*-value decreased, while a^*-value increased as fermentation time increased ($p < 0.05$). This was correlated well with the increase in browning intensity (A_{420}) ($p < 0.05$). ATP was not detected in raw material, whereas adenosine diphosphate (ADP) and adenosine monophosphate (AMP) disappeared after salting and drying, respectively. After 30 days of fermentation, *Kapi* contained hypoxanthine (Hx) (112.49 mg/100 g dry sample) as the most abundant ATP-related compound. Aldehydes, ketones as well as *N*-containing compounds increased as fermentation time increased ($p < 0.05$). *N*-containing compounds, mainly pyrazine derivatives, were prevalent volatiles in *Kapi*. Moreover, antioxidative activities increased, particularly when fermentation proceeded, as evidenced by the increases in 2,2-diphenyl-1-picrylhydrazyl (DPPH) and 2,2-azino-bis (3-ethylbenzthiazoline-6-sulphonic acid) (ABTS) radical scavenging activities, and ferric reducing antioxidant power (FRAP) ($p < 0.05$). According to these results, several changes of *Kapi* took place throughout all processes. However, odorous compounds and flavorants were mainly formed during fermentation process. Those compounds were plausibly involved in the unique flavor/taste of *Kapi.*

9.2 Introduction

Kapi is the traditional salted shrimp paste of Thailand. It is purplish pink to dark brown in color with strong odor and paste-like consistency. Being served as the seasoning ingredient in many Thai dishes, the quality associated with odor, flavor as well as color has been considered important. Generally, *Kapi* is made from krill or small sized shrimps. Shrimp belonging to *Acetes vulgaris*, by-products from commercial fishing, shows the high potential as alternative raw material due to its high availability, especially in the southern part of Thailand (Pongsetkul *et al.,* 2015a). Overall quality of *Kapi* produced from this shrimp species was equivalent to the commercial *Kapi* produced from krill (*Mesopododsis* spp.) (Pongsetkul *et al.,* 2015b). *Kapi* is generally made by mixing the raw material with salt and sun-drying for 2 days, followed by grinding into a fine paste. Fermentation is allowed to take place in earthen jar for at least 1 month or until the desirable odor is generated (Pongsetkul *et al.,* 2014). Several compounds including short chain peptides, free amino acids, adenosine triphosphate (ATP)-degradation products, volatile compounds, etc, have a profound effect on the sensory characteristics of Philippine salt-fermented shrimp paste (Peralta *et al.,* 2008).

Maillard reaction or non-enzymatic browning reaction is related with the development of color, aroma compounds as well as bioactive compounds, especially antioxidants (Benjakul *et al.,* 2005; Wijewickreme *et al.,* 1997). Melanoidins, the final Maillard reaction products (MRPs), also have a wide range of activity such as antioxidant, antimicrobial and metal chelating activity (Morales *et al.,* 2005). MRPs have been reported to contribute to color of *Kapi* (Peralta *et al.,* 2008). Flavor is one of the most important factors governing *Kapi* quality. Several volatile components of this product associated with its flavor have been reported (Cha and Cadwallader, 1995; Pongsetkul *et al.,* 2014; Wittanalai *et al.,* 2011). Nucleotides, ATP and its related compounds including adenosine diphosphate (ADP), adenosine monophosphate (AMP), inosine monophosphate (IMP), inosine (HxR) as well as hypoxanthine (Hx), also play the role in taste or flavor of many marine products, especially for umami or sweet-taste (Kim and Lee, 2008). Hayashi *et al.* (1978) reported that the existence of IMP or AMP together with glutamic acid showed a distinct taste-enhancing effect in dried bonito. Although *Kapi* could be produced using shrimp *A. vulgaris* and showed

the quality equivalent to *Kapi* produced from krill (Pongsetkul *et al.,* 2015a), the changes in particular components, especially those contributing to taste and flavor as well as bioactivities during processing and fermentation of this *Kapi*, have not been reported.

9.3 Objective

To monitor the changes in some components including volatile compounds, MRPs as well as ATP-related compounds of *Kapi* produced from *A. vulgaris* and to examine antioxidative activities during *Kapi* production including salting, drying as well as fermentation processes.

9.4 Materials and methods

9.4.1 Sample collection

Ten kg of fresh shrimp *A. vulgaris* (average body length: 15.6±1.4 mm; average weight: 41.3±9.8 mg, n=20), used as the composite sample, were obtained from a local market in Ko-yo, Songkhla province, Thailand. Shrimp were transported directly to the laboratory in a polystyrene box containing ice using a shrimp/ice ratio of 1:2 (w/w) within approximately 2 h.

9.4.2 Preparation of *Kapi*

Upon arrival, shrimp were washed with 3% NaCl solution, drained and placed into the basket [R]. Shrimp were mixed together with salt at the ratio of 5:1 (w/w). The mixture was transferred to the polypropylene basket and covered with cheese cloth. The mixture was allowed to stand at room temperature (28-30°C) overnight [S]. Salted shrimp were drained and ground to uniformity using a blender (National, Tokyo, Japan). Salted shrimp were dried with sunlight by spreading out on the fiberglass mats. Drying step were continued until the moisture content of dried shrimp was in the range of 35-40% [D] as determined by AOAC method (AOAC, 2000). Subsequently, dried shrimp were impacted into earthen jars, covered with plastic bag tightly (close system) and fermentation was proceeded at room temperature (28-

30°C) for 1 month. During fermentation, samples were taken at day 10 [F1], 20 [F2] and 30 [F3]. All six samples during *Kapi* processing/fermentation were used for analyses.

9.4.3 Changes in compositions and properties during *Kapi* processing/ fermentation

9.4.3.1 Changes in color

Color of samples was determined using a colorimeter (ColourFlex, Hunter Lab Reston, VA) as described by Pongsetkul *et al.* (2014). The results were recorded in the CIE system and expressed as L^* (lightness), a^* (redness/greenness), b^* (yellowness/blueness), ΔE^* (total difference of color) and ΔC^* (the difference in chroma).

9.4.3.2 Changes in browning index and MRPs

- Preparation of water extract

Water extract was prepared according to the method of Peralta *et al.* (2008) with a slight modification. Sample (2 g) was mixed with 50 ml of distilled water. The mixtures were homogenized at a speed of 11,000 rpm for 2 min using a PT 2100 homogenizer (KinematicaAG, CH-6014, Littau/Luzern, Switzerland). The homogenates were then subjected to centrifugation at 8000×*g* for 15 min at room temperature using a RC-5B plus centrifuge (Sorvall, Norwalk, CT, USA). The supernatant was collected and adjusted to 50 ml using distilled water.

- Measurement of intermediate MRPs

Non-fluorescent and fluorescent intermediate products from Maillard reaction in the water extract were determined as per the method of Pongsetkul *et al.* (2015b). The absorbance of the appropriately diluted extract was measured at 280 and 295 nm using the UV-1601 spectrometer (Shimadzu, Kyoto, Japan) to monitor the formation of non-fluorescent intermediate products. For the fluorescent intensity, appropriately diluted extract was measured at an excitation wavelength of 347 nm and

emission wavelength of 415 nm using a fluorescence spectrophotometer RF-1501 (Shimadzu, Kyoto, Japan).

- Measurement of browning intensity

The browning intensity of the extract was determined following the method of Pongsetkul *et al.* (2015b). Appropriate dilution of water extract was made using distilled water and the absorbance was read at 420 nm using the UV-1601 spectrometer.

9.4.3.3 Changes in ATP-related compounds

ATP and its related compounds of samples were determined using HPLC as per the method of Kuda *et al.* (2007) with a slight modification. Firstly, sample (2 g) was mixed with 10 ml of 10% perchloric acid and then centrifuged at 8000×*g* for 5 min at 4°C. The supernatant was collected. The pellet was re-extracted in the same manner. The supernatants were combined and adjusted to 25 ml using 10% perchloric acid. Then, the mixture was neutralized using KOH and the precipitate was removed by centrifugation as described above. The supernatants were filtered (Millex-LG 0.20 μm) and the filtrates were subjected to analysis using HPLC (Hitachi L2130, Hitachi koki Co., Ltd., Tokyo, Japan) equipped with a column (Shodex GS-320 HQ, Showa Denko K.K., Tokyo, Japan). The condition included mobile phase: 200 mM $NaH_2PO_4 \cdot 2H_2O$, flow rate: 0.6 ml/min, and temperature: 30°C. Detector (Hitachi L7420, Hitachi koki Co., Ltd., Tokyo, Japan) was used and the absorbance of 260 nm was monitored. ATP-related compounds of sample during *Kapi* processing/ fermentation were expressed as mg/100g dry sample.

9.4.3.4 Changes in volatiles

Volatile compounds of samples were determined using a solid-phase microextraction gas chromatography mass spectrometry (SPME GC-MS) as described by Pongsetkul *et al.* (2015a). The identified volatile compounds were expressed in the terms of relative abundance.

9.4.3.5 Changes in antioxidative activities

Water extracts of samples were prepared as described above. Prior to assay, the extracts were approximately diluted using distilled water. 2,2-diphenyl-1-picrylhydrazyl (DPPH) and 2,2-azino-bis (3-ethylbenzthiazoline-6-sulphonic acid) (ABTS) radical scavenging activities, as well as ferric reducing antioxidant power (FRAP) were determined according to the method of Faithong and Benjakul (2012). Activities were expressed as μmol Trolox equivalents (TE)/g sample.

9.4.4 Statistical analysis

All experiments were conducted in triplicate using there different lots of samples. Statistical analysis was done by using one-way analysis of variance (ANOVA). Mean comparison was conducted using the Duncan's multiple range test (Steel *et al.,* 1980). Analysis was performed using the SPSS package (Version 10.0) (SPSS for windows, SPSS Inc., Chicago, II, USA).

9.5 Results and discussion

9.5.1 Changes in color

Changes in color of *Kapi* during processing and fermentation were observed as reported in Table 26. L^*-value (lightness) of raw shrimp was 65.62. The lightness of shrimp was decreased after salting overnight ($p < 0.05$). With high salt (about 20%, w/w), dehydration of shrimp occurred via “salting out” effect. After drying, the pigments in shrimp, particularly astaxanthin, became more concentrated. This was shown by the increases in a^*- and b^*-values. During fermentation, the continuous decrease in L^*-value was obtained up to 30 days of fermentation ($p < 0.05$). This was coincidental with the increases in a^*- and b^*-values of sample as fermentation proceeded ($p < 0.05$). Increases in both ΔE^* and ΔC^* were noticeable as fermentation time increased. During fermentation, carotenoproteins were more likely degraded, induced by both indigenous and microbial proteases. As a consequence, free carotenoids, particularly astaxanthin, were liberated. Appearance of red color in shrimp was caused by free carotenoids, especially astaxanthin (Chantarasuwa *et al.,* 2011).

Astaxanthin is a pigment commonly found in crustacean, providing the tissue with red-orange pigmentation (Higuera-Ciapara *et al.,* 2006). During fermentation, the slight decrease in *b**-value was observed as fermentation time increased ($p < 0.05$). Overall, *ΔE**-value was increased along with the processes used, salting and drying. Also, the values were increased with increasing fermentation time. Dissaraphong *et al.* (2006) also found the decreases in *L** and *b**-value, but the increase in *a**-value during extended fermentation time of fish sauce production, indicating the development of brown color. Kim *et al.* (2004) found that the lightness (*L**) and yellowness (*b**) of salted and fermented anchovy sauce decreased during storage up to 6 months. In the present study, the decreases in *L**-value and increases in *ΔE**-value of *Kapi* after fermentation were coincidental with brownish red/purple in color. Faithong and Benjakul (2012) also revealed that the lightness of *Kapi* was gradually decreased and became darker during storage up to 12 months. Peralta *et al.* (2008) reported that the brown color of shrimp pastes, as an index of the formation of MRP, showed significant increases with increasing fermentation time. Overall, changes in color occurred at all steps of *Kapi* production, particularly during fermentation.

Table 26. Changes in color during processing/fermentation of *Kapi*

Samples*	Color				
	*L**	*a**	*b**	Δ*E**	Δ*C**
R	$65.62{\pm}0.22^{a}$	$-1.32{\pm}0.09^{f}$	$0.29{\pm}0.03^{e}$	$27.93{\pm}0.22^{f}$	$0.44{\pm}0.09^{d}$
S	$55.63{\pm}0.31^{b}$	$0.47{\pm}0.02^{e}$	$-0.58{\pm}0.06^{f}$	$37.95{\pm}0.31^{e}$	$-0.17{\pm}0.05^{e}$
D	$53.44{\pm}0.28^{c}$	$3.60{\pm}0.35^{d}$	$18.38{\pm}0.26^{a}$	$44.19{\pm}0.23^{c}$	$17.81{\pm}0.31^{a}$
F1	$55.14{\pm}0.09^{b}$	$6.22{\pm}0.07^{c}$	$15.44{\pm}0.42^{b}$	$41.85{\pm}0.08^{d}$	$15.73{\pm}0.40^{c}$
F2	$50.52{\pm}0.10^{d}$	$8.21{\pm}0.03^{b}$	$15.00{\pm}0.03^{c}$	$46.34{\pm}0.08^{b}$	$16.18{\pm}0.03^{b}$
F3	$48.02{\pm}0.09^{e}$	$10.16{\pm}0.08^{a}$	$13.44{\pm}0.14^{d}$	$48.63{\pm}0.11^{a}$	$15.94{\pm}0.14^{bc}$

*R: raw material; S: after salting; D: after drying; F1, F2 and F3: after fermentation for 10, 20 and 30 days.
Mean ± SD from triplicate determinations. Different lowercase superscripts in the same column indicate the significant difference ($p < 0.05$).

9.5.2 Changes in MRPs and browning intensity

Intensity of intermediate products as determined by both non-fluorescent intensity (A_{280}, A_{295}) and fluorescence intensity as well as browning

intensity (A_{420}) of *Kapi* during processing/fermentation were monitored as shown in Figure 25. From Figure 25A, continuous increase in A_{280} was found at every steps of *Kapi* production and reached the value of 0.82 when salted shrimp were subjected to fermentation for 30 days ($p < 0.05$). However, there was no difference in A_{280} between raw material (R) and salted sample (S) ($p < 0.05$). For A_{295}, the increases during salting, drying and fermentation up to 20 days were also found. Nevertheless, slight decrease in A_{295} was observed after 30 days of fermentation ($p < 0.05$) as depicted in Figure 25B. In general, A_{280} and A_{295} have been used to determine the formation of non-fluorescent intermediate compounds of the Maillard reaction (Ajandouz *et al.,* 2001). The increases in these values suggested the continuous formation of an uncolored compound, which could be the precursor of the Maillard reaction (Benjakul *et al.,* 2005). Protein degradation occurring during *Kapi* production could provide higher amount of free amino groups, which were able to undergo the Maillard reaction to a higher extent during processing and the extended fermentation.

The fluorescence intensity of shrimp was decreased ($p < 0.05$) when shrimp was salted overnight as shown in Figure 25C. However, the values increased sharply when subjected to drying ($p < 0.05$). The fluorescence intensity was still increased during the first 10 days of fermentation. Nevertheless, the decreases in fluorescence intensity were found with increasing fermentation time (20-30 days) ($p < 0.05$). The results suggested the conversion of those intermediates to the final brown products. Development of fluorescent compounds occurs in the Maillard reaction prior to the generation of brown pigments (Dissaraphong *et al.,* 2006). Fluorescent compounds have been known as the precursors of brown pigments (Benjakul *et al.,* 2005).

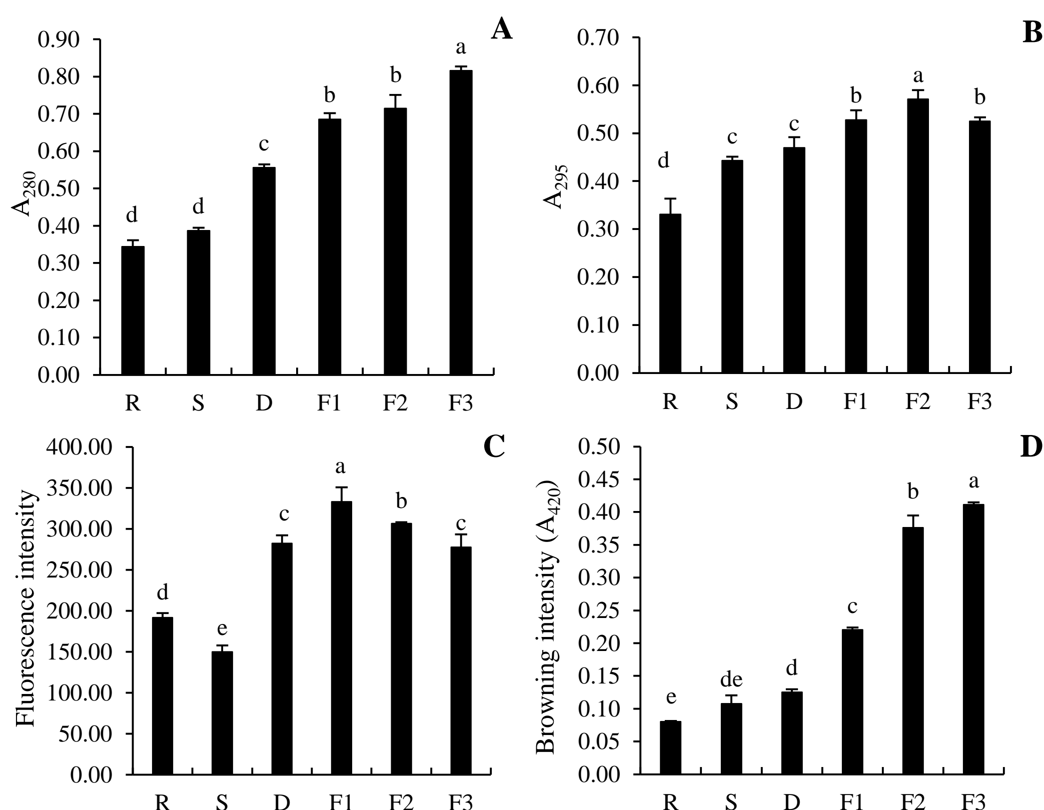

Figure 25. Changes in A_{280} (A), A_{295} (B), fluorescence intensity (C) and browning intensity (A_{420}) (D) during processing/fermentation of *Kapi*. R: raw material; S: after salting; D: after drying; F1, F2 and F3: after fermentation for 10, 20 and 30 days.

Bars represent the standard deviation from triplicate determinations. Different letters on the bars denote the significant differences ($p < 0.05$).

The browning intensity of shrimp paste was gradually increased throughout *Kapi* processing as shown in Figure 25D. Slight increase in A_{420} was found after drying ($p < 0.05$). With increasing fermentation time, the marked increase in A_{420} was observed ($p < 0.05$) and reached the value of 0.41 at day 30 of fermentation. The increase in A_{420} was used as an indicator for browning development in the final stage of the browning reaction (Ajandouz *et al.,* 2001). This was in agreement with the decrease in *L**-value of *Kapi* after fermentation (Table 26). During processing/ fermentation, enzymatic browning reactions, induced by polyphenoloxidase (PPO) from raw material, plausibly occurred. PPO has been known to induce the hydroxylation of phenols with subsequent polymerization, in which melanin is formed (Nirmal and Benjakul, 2009). Maillard reaction was found to be responsible for the

brown color in fermented products (Dissaraphong *et al.,* 2006). Most of nitrogenous compounds generated, particularly during fermentation, are free amino acids and small peptides, which contributed to brown color development via Maillard reaction (Dissaraphong *et al.,* 2006). Carbohydrate derivatives, such as glucose-6-phosphate and other substances present in the metabolic pathways, could also act as reactants in the Maillard reaction (Kawashima and Yamanaka, 1990). Moreover, oxidation products, such as aldehyde were able to react with free amino acids, liberated during fermentation. Therefore, processing or fermentation process directly influenced the color development of *Kapi*. Overall, *Kapi* became browner in color when fermentation proceeded. The color of *Kapi* is the one factor affecting the overall consumer acceptance as described by Pongsetkul *et al.* (2015a).

9.5.3 Changes in ATP-related compounds

Table 27 shows the changes in ATP-related compounds during processing/fermentation of *Kapi.* In the present study, ATP was not detected in all samples during *Kapi* processing/fermentation. In fresh shrimp, the content of ADP was low (14.30 mg/100 g dry sample), while AMP and IMP were dominant (52.82 and 52.56 mg/100 g dry sample, respectively), suggesting a rapid conversion of ATP into AMP and IMP of shrimp after death, particularly during transportation. Furthermore, low amounts of HxR and Hx were found in fresh sample (8.44 and 0.58 mg/100 g dry sample, respectively). When shrimp were subjected to salting overnight, followed by sun-drying, the sharp decrease in AMP was noted with coincidental increase in IMP ($p < 0.05$). HxR and Hx were obviously increased after salting and drying, suggesting that AMP and IMP were continously degraded with coincidental formation of HxR and Hx. After the death, ATP in fish is rapidly hydrolyzed to IMP by the action of endogenous enzyme (Gram and Huss, 1996). The subsequent degradation of IMP to HxR and Hx is accerelated with the participation of autolytic and microbial enzymes as well as the storage temperature. During the iced storage, degradation of IMP to HxR is much slower than that occurred at ambient temperature. Salting and drying were carried out at the temperature range of 25-40°C. Therefore, IMP was degraded to HxR rapidly with concomitant increases in Hx and HxR levels. After drying, IMP was predominat

Table 27. Changes in ATP-related compounds during processing/fermentation of *Kapi*

ATP-related compounds (mg/ 100 g dry sample)	Samples*					
	R	S	D	F1	F2	F3
ATP	ND	ND	ND	ND	ND	ND
ADP	14.30±1.10a	2.33±0.37b	ND	ND	ND	ND
AMP	52.82±1.82a	34.54±1.93b	4.41±1.04c	ND	ND	ND
IMP	52.56±3.05c	85.76±2.04a	78.67±3.01b	10.55±1.41d	ND	ND
Inosine (HxR)	8.44±0.51e	5.50±0.46f	21.87±0.18c	76.13±1.33a	50.10±0.65b	16.02±1.19db
Hypoxanthine (Hx)	0.58±0.09e	0.46±0.06e	23.85±2.11d	41.57±1.09c	77.98±0.83b	112.49±1.84ba

*R: raw material; S: after salting; D: after drying; F1, F2 and F3: after fermentation for 10, 20 and 30 days.
Mean ± SD from triplicate determinations. Different lowercase superscripts in the same row indicate the significant difference ($p < 0.05$).

(78.67 mg/100 g dry sample) and was intensively decreased and totally disappeared when fermentation proceeded up to 20 days. Conversely, the increases in HxR and Hx were observed ($p < 0.05$). HxR was sharply increased within the first 10 days of fermentation and then decreased as fermentation time increased ($p < 0.05$). However, the gradual increase in Hx content was found throughout fermentation of 30 days and reached the value of 112.49 mg/100 g dry sample at day 30 of fermentation. The result revealed that Hx was the most abundant ATP-related compound found in the final *Kapi*. Changes in ATP-related compounds in some fermented products have been reported. Kim and Lee (2008) found that ATP, ADP and IMP were not detected in soy sauce during fermentation process. Only Hx was found in this product. Hx was the dominant ATP-related compounds in traditional Korean soy sauce made by enzymatic hydrolysis, however, Hx was slightly decreased when fermentation time was more than 3 months (Park and Sohn, 1997). Overall, ATP-related compounds were found to be varied during processing and fermentation. Those compounds contributed to the taste and flavor of *Kapi.* Shahidi (1994) reported that free amino acids and nucleotides are the most important components contributing to the desirable flavor of seafoods. The contribution of 5´nucleotides (5´-IMP and 5´-GMP) to the umami taste of seafood is well recognized. However, Hx gives a bitter taste (Shahidi, 1994). HxR and Hx in *Kapi* along with other compounds such as free amino acid, short-chain peptides, as well as volatile compounds such as *N*-containing compounds, etc., were plausibly involved in the unique flavor/taste in *Kapi.*

9.5.4 Changes in volatile compounds

During *Kapi* production, totally 51 volatile compounds including aldehydes (6), ketones (8), alcohols (12), nitrogen-containing compounds (10), sulfur-containing compounds (3), hydrocarbons (6) and others (6) were identified (Table 28). Degradation of proteins, lipolysis, lipid oxidation, Maillard reaction, interaction between MRPs with lipid-oxidized products and other reactions more likely contributed to the formation of volatile compounds, which determined the odor or flavor of *Kapi*.

Table 28. Changes in volatile compounds during processing/fermentation of *Kapi*

	Volatile compounds	Peak area (Abundance) × 10^5					
		R	S	D	F1	F2	F3
Aldehydes	3-methyl-butanal	ND	ND	83.33	40.08	55.53	60.53
	Pentanal	ND	ND	ND	9.91	15.16	18.01
	2-hexanal	96.66	34.12	10.88	4.06	4.33	9.22
	Heptanal	ND	ND	3.54	28.88	40.66	16.16
	2-octenal	88.92	18.22	ND	ND	ND	ND
	Benzaldehyde	206.66	114.32	259.32	199.19	203.11	225.33
Ketones	1-phenyl-ethanone	34.91	23.91	23.33	16.12	44.09	67.66
	1,2-diphenyl-ethanone	9.97	12.55	ND	2.55	ND	ND
	1-(2-aminophenyl)-ethanone	ND	ND	8.87	16.17	88.22	82.15
	2-pentanone	ND	ND	ND	ND	10.12	15.99
	2-hexanone	45.43	55.50	50.66	41.29	69.69	55.63
	2-heptanone	16.16	20.08	33.45	65.65	66.67	109.83
	3-octanone	66.62	ND	2.11	ND	ND	ND
	3,5-octadiene-2-one	106.63	24.44	55.32	ND	ND	ND
Alcohols	Benzenemethanol	16.23	77.61	45.40	98.66	204.11	240.44
	2-butyl-ethanol	ND	10.21	8.09	9.22	23.23	56.55
	2-methyl-1- propanol	142.11	98.77	84.44	105.56	123.55	133.29
	1-butanol	16.67	ND	ND	ND	ND	ND
	3-methyl-butanol	105.66	100.41	122.21	99.56	86.47	44.03
	1-pentanol	ND	ND	ND	6.67	15.55	103.24
	1-penten-3-ol	145.66	134.56	69.96	105.55	102.02	66.98
	5-methoxy-1-pentanol	ND	ND	13.93	105.66	225.59	99.97
	1-hexanol	25.55	ND	ND	ND	ND	359.95
	2-ethyl, 1-hexanol	105.66	10.97	ND	ND	ND	ND
	2,7-octadience-1-ol	16.63	15.55	3.35	6.67	ND	ND
	1-octen-3-ol	ND	ND	45.23	99.98	103.45	266.57
N-containing compounds	Methyl-pyrazine	16.66	2.67	8.81	59.53	89.99	205.46
	2-ehtyl-6-methyl-pyrazine	206.66	199.87	255.56	255.92	301.45	33.25
	3-ethyl-5-methyl-pyrazine	98.96	45.33	78.10	102.22	106.66	112.46
	2,3-diethyl, 5-methyl-pyrazine	ND	ND	ND	10.18	55.65	143.15
	2,5-dimethyl-pyrazine	255.59	105.44	295.16	334.99	356.69	505.15
	2,6-dimethyl-pyrazine	98.99	161.22	215.66	269.99	350.67	321.80
	3-ethyl-2,5-dimethyl-pyrazine	404.55	415.55	239.39	446.41	355.99	466.51
	2-ethyl-3,5-dimethyl-pyrazine	98.99	15.66	105.68	222.56	505.51	413.51
	Trimethyl-pyrazine	ND	ND	14.45	56.65	66.22	125.67
	2,3,5-trimethyl-6-ethyl-pyrazine	ND	19.09	20.04	55.69	ND	ND

*R: raw material; S: after salting; D: after drying; F1, F2 and F3: after fermentation for 10, 20 and 30 days.
ND: non-detectable.

Table 28. Changes in volatile compounds during processing/fermentation of *Kapi* (Cont.)

	Volatile compounds	Peak area (Abundance) × 10^5					
		R	S	D	F1	F2	F3
S-com**	Dimethyl-disulfide	106.67	54.56	98.10	22.45	ND	ND
	Dimethyl-trisulfide	65.32	10.14	ND	ND	ND	ND
	Methamethiol	225.46	198.98	103.55	105.66	104.42	96.52
Hydrocarbons	2,6,10,14-tetramethyl-pentadecan	ND	ND	ND	ND	23.22	45.45
	3-tetradocene	6.65	ND	19.22	15.51	50.66	22.38
	2,3-butanediene	ND	ND	3.59	12.99	ND	26.63
	2-undecane	56.79	55.13	26.66	25.53	19.22	44.05
	Hexadecane	19.02	23.44	4.12	ND	ND	ND
	2,6-cyclohexadien	44.56	50.56	21.29	12.33	ND	ND
Others	Propanoic acid	ND	ND	ND	ND	6.67	19.93
	Butanoic acid	ND	25.56	96.26	77.61	25.22	10.12
	Octanoic acid	ND	ND	18.87	22.55	ND	ND
	Methyl-ester-butanoic acid	ND	ND	55.23	59.66	82.46	102.12
	Phenol	202.44	205.11	255.65	203.05	201.11	221.21
	1H-Indole	95.87	95.53	105.55	205.99	261.63	411.48
	Total intensity	**3249.31**	**2435.06**	**2964.36**	**3638.90**	**4444.99**	**5358.38**

*R: raw material; S: after salting; D: after drying; F1, F2 and F3: after fermentation for 10, 20 and 30 days.
ND: non-detectable. **S*-com: Sulfur containing compounds.

Six aldehydes were found in samples during *Kapi* processing/ fermentation. Aldehydes are the most prominent volatiles produced during lipid oxidation and have been used successfully to monitor lipid oxidation in a number of foods (Sae-leaw *et al.,* 2013). For fresh shrimp, only 3 aldehydes were observed, including 2-hexanal, 2-octenal and benzaldehyde. 2-octenal was decreased after being salted and totally disappeared after drying step. 2-octenal wasfound in fresh salmon (Varlet and Fernandez, 2010). It is a product of oxidation of n-6 PUFA such as arachidonic acid, which gives green aromatic notes. Conversely, 3-methyl-butanal, pentanal and heptanal were not detected in fresh shrimp, but these aldehydes were generated during processing/fermentation. 3-methylbutanal are potent flavoring compounds, which were responsible for meaty note in fermented foods such as *Ishiru* (Japanese fish sauce) (Michihata *et al.,* 2002), tuna sauce (Cha and Cadwallader, 1998) and cheese (Magboul and McSweeney, 1999). Benzaldehyde was found as a major

aldehyde in all samples. It was reported to have a pleasant almond, nutty and fruity aroma (Cha and Cadwallader, 1995). Thus, aldehydes were the products derived from lipid oxidation, taken place during fermentation**.**

Changes in ketones were also found during *Kapi* processing/ fermentation. 1-phenyl-ethanone, 2-hexanone and 2-heptanone were found in fresh shrimp and still remained in *Kapi* after 30 days of fermentation. Some ketones, 1,2-diphenyl-ethanone, 3-octanone and 3,5-octadiene-2-one, were observed in fresh shrimp but disappeared during processing. However, some ketones were generated during processing/fermentation and still remained in the final product. Those included 1-(2-aminophenyl)-ethanone and 2-pentanone. In general, both aldehydes and ketones were more likely generated from lipid oxidation during fermentation (Takeungwongtrakul *et al.,* 2012). Ketones seemed to be responsible for the cheesy note in fish sauce odor as reported by Peralta *et al.* (1996). It was generated by microbial enzymatic actions on lipids and/or amino acids, or by the Maillard reaction. Although the flavor notes of ketones are generally desirable, their aroma contributions might be minimal. Ketones generally have high odor threshold values. Additionally, low levels of these compounds were formed in *Kapi* product (Cha and Cadwallader, 1995).

Various alcohols were found in *Kapi* during processing/fermentation. Among 12 alcohols, only 4 alcohols including benzenemethanol, 2-methyl-1-propanol, 3-methyl-butanol and 1-penten-3-ol were detected in fresh shrimp and still remained in the final product. Some alcohols, including 1-butanol, 2-ethyl-hexanol and 2,7-octadience-1-ol, were detected in fresh shrimp and were drastically lost during processing. 1-butanol was also found in fresh shrimp *Parapenaeus longirostris* (Giogios *et al.,* 2013). 2-butyl-ethanol, 1-pentanol, 5-methoxy-1-pentanol and 1-octen-3-ol were generated during *Kapi* processing/fermentation. 1-octen-3-ol is an important contributor to off-flavor due to its low odor threshold and it is formed from oxidation of arachidonic acid by lipoxygenase (Varlet and Fernandez, 2010). Thiansilakul *et al.* (2011) reported that 1-octen-3-ol contributed to the strong intensities of fishy and rancid off odors in washed Asian seabass mince. Overall, intensity of alcohols increased as fermentation time increased. Alcohols are known as the secondary products generated by the decomposition of hydroperoxides of fatty acid (Sae-leaw *et al.,* 2013). This was coincidental with our previous study, in which lipid oxidation in *Kapi* increased during

fermentation period of *Kapi* (Pongsetkul *et al.,* 2015a). However, alcohols found in *Kapi* were quite low in abundance. Furthermore, they might not have a paramount impact on *Kapi* flavor because of their high flavor thresholds (Cha and Cadwallader, 1995).

Nitrogen-containing compounds were the major compounds found in *Kapi*, which were all pyrazine derivatives. In general, the intensity of these compounds increased throughout production, especially as the fermentation time increased. Seven *N*-containing compounds were identified in fresh shrimp and were still found throughout the process. Those included methyl-pyrazine, 3-ethyl-5-methyl-pyrazine, 2,5-dimethyl-pyrazine, etc. It was noted that 2,3-diethyl-5-methyl-pyrazine and trimethyl-pyrazine were obtained during processing and increased when fermentation time increased. Only 2,3,5-trimethyl-6-ethyl-pyrazine was generated at the beginning of fermentation and disappeared during the extended fermentation. Shibamoto (1989) reported that pyrazines, with low flavor thresholds, have been known to have both positive and negative impacts on the flavor quality of cooked foods. However, many previous studies reported that pyrazine derivatives seem to give desirable odor or flavor for several fermented seafoods including *Ishiru* (Japanese fish sauce) (Michihata *et al.,* 2002), *Noucmam* (Vietnamese fish sauce) (Lopetcharat *et al.,* 2001), fermented dried shrimp (*Acetes chinensis*) (Lu *et al.,* 2011) as well as *Kapi* (Cha and Cadwallader, 1995; Pongsetkul *et al.,* 2015a). Pyrazines have been reported to contribute to nutty, roasted and toasted characteristics in roasted shrimp (Kubota *et al.,* 1986), and burnt and sweet odors in fish sauce (Lopetcharat *et al.,* 2001). These compounds are probably generated via Maillard and pyrolysis reactions through Strecker degradations from various nitrogen sources such as amino acids in heat processed foods (Lu *et al.,* 2011). *N*-containing compounds in *Kapi* could therefore be generated, thus affecting flavor and odor of the final product to some extent.

Three *S*-containing compounds were found in fresh shrimp. During *Kapi* production, all these compounds were drastically decreased. Dimethyl-disulfide and dimethyl-trisulfide were not obtained in the final product, while methamethiol was still remained at day 30 of fermentation. *S*-containing compounds were found in fresh shrimp (*Parapenaeus longirostris*) (Varlet and Fernandez, 2010), crab (*Charybdis feriatus*) (Chung, 1999), squid (*Loligo vulgaris*) (Kawai *et al.,* 1991). The *S*-containing

compounds are considered to be enzymatically produced in seafood from methionine and cysteine (Varlet and Fernandez, 2010). Total disappearance or low abundance of *S*-containing compounds in *Kapi* at the end of process might be due to the loss in activities of *S*-containing compound producing-enzymes, both endogenous and microbial enzymes, or the degradation of these compounds into others.

2,6,10,14-tetramethyl-pentadecane, 3-tetradocene, 2,3-butanediene and 2-undecane were found in the final *Kapi*. Hexadecane and 2,6-cycloehexadien were obtained in only fresh sample, but were not found in *Kapi*. Latorre-Moratalla *et al.* (2011) reported that these alkanes and alkenes are mainly formed from auto-oxidation of fatty acids released from triglycerides. Due to low abundance, these compounds seemed not to be the major contributor to flavor characteristics of *Kapi*.

For other compounds, three acids were obtained in *Kapi* after 30 days of fermentation. Those included propanoic acid, butanoic acid and methyl-ester-butanoic acid. Kubota *et al.* (1986) reported that butanoic acid gave the cheesy notes in roasted shrimp. Octanoic acid, which was reported to contribute to oily notes (Chung *et al.,* 1999), was generated during processing but disappeared in *Kapi*. Phenol was found in fresh shrimp with high intensity and remained constant at the end of process. Phenol was reported to give an undesirable aroma in seafood (Cha and Cadwallader, 1995). Furthermore, the increases in indole intensity were observed throughout *Kapi* production, especially during fermentation. Indole is the degradation product from tryptophan (Pongsetkul *et al.,* 2015b; Lakshmanan *et al.,* 2002). The result suggested that some nutritional loss associated with the lower tryptophan content plausibly occurred in *Kapi*.

Overall, changes in volatile compounds were observed during *Kapi* processing/fermentation. Aldehydes, ketones as well as *N*-containing compounds were drastically increased as fermentation time increased. Protein and lipid degradation/ decomposition during fermentation might be associated with flavor or odor of *Kapi*.

9.5.5 Changes in antioxidative activities

DPPH radical scavenging activities of water extract of shrimp during *Kapi* production are depicted in Figure 26A. DPPH radical scavenging assay has been widely used to evaluate antioxidant properties of compounds as free radical scavengers

or hydrogen donors (Klompong *et al.,* 2007). Water extract of fresh shrimp had DPPH radical scavenging activity of 2.71 μmol TE/g dry sample. There was no change in activity between the samples subjected to salting and drying processes ($p > 0.05$). However, the sharp increase in DPPH radical scavenging activity was observed during fermentation period and reached the value of 10.06 μmol TE/g dry sample at day 30 of fermentation. It was suggested that peptides or free amino acids generated during fermentation process had the ability to donate the hydrogen atom to free radicals, in which the propagation could be retarded. For fermented products, hydrolysis was generally progressed throughout the prolonged fermentation, leading to the increases in hydrolyzed peptides and amino acids. Low molecular weight peptides and amino acids have been reported to possess antioxidant activity (Binsan *et al.,* 2008; Faithong *et al.,* 2010; Sae-leaw *et al.,* 2013).

ABTS radical scavenging activity of water extract of shrimp during *Kapi* processing/fermentation was also monitored as shown in Figure 26B. ABTS assay is an excellent tool for determining both hydrophilic and lipophilic antioxidants, which are able to scavenge aqueous phase radicals and lipid peroxyl radicals (Sun and Tanumihardjo, 2007). ABTS radical scavenging activity of fresh shrimp was 4.68 μmol TE/g dry sample. Slight increases were obtained after salting and drying processes ($p < 0.05$). With similar trend to DPPH radical scavenging activity, ABTS radical scavenging activity markedly increased during fermentation ($p < 0.05$). At day 30 of fermentation, water extract of sample had ABTS radical scavenging activity of 18.13 μmol TE/g dry sample. Apart from antioxidant peptides in *Kapi*, other antioxidative compounds including MRPs were present (Pongsetkul *et al.,* 2015b). Increases in activity were correlated well with the increase in MRPs during *Kapi* production, especially during fermentation (Figure 25).

For FRAP, water extract showed the increase in FRAP after drying. It was noted that the highest activity was observed at day 30 of fermentation ($p < 0.05$). FRAP of final product was 15.96 μmol TE/g dry sample. In general, FRAP is used for measurement of the reducing ability (TPTZ-Fe^{3+} to TPTZ-Fe^{2+}) (Pongsetkul *et al.,* 2015b). The reducing ability of *Kapi* was possibly due to the presence of peptides, which donated electrons to free radicals, leading to the prevention or retardation of propagation. *Kapi* has been reported to possess FRAP (Faithong *et al.,* 2010;

Pongsetkul *et al.,* 2015b). It was suggested that *Kapi* could be the good source of natural antioxidants with different modes of action in prevention of lipid oxidation.

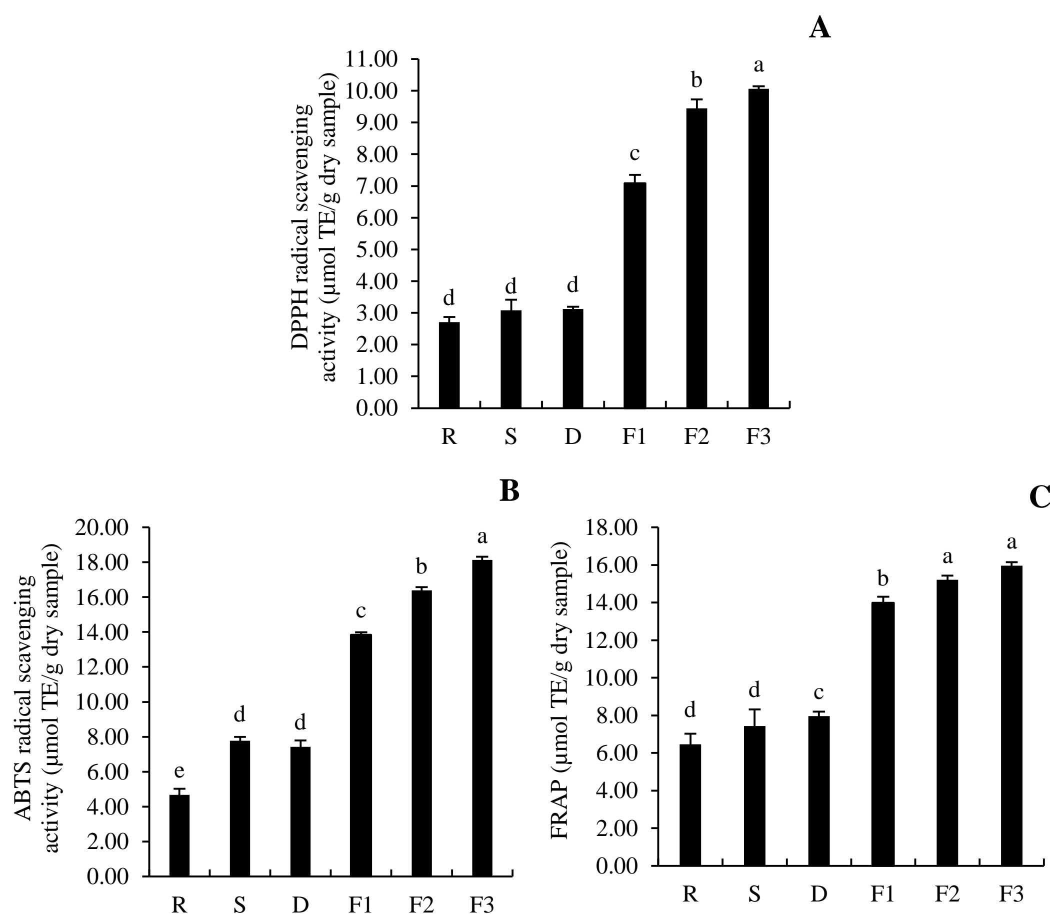

Figure 26. Changes in DPPH radical scavenging activity (A), ABTS radical scavenging activity (B) and FRAP (C) during processing/fermentation of *Kapi*. R: raw material; S: after salting; D: after drying; F1, F2 and F3: after fermentation for 10, 20 and 30 days. Bars represent the standard deviation from triplicate determinations. Different letters on the bars denote the significant differences ($p < 0.05$).

9.6 Conclusion

Properties of *Kapi*, salted shrimp paste, was governed by all processes, especially fermentation. Color generally turned to be browner or darker as fermentation time was extended. Degradation of ATP and its related compounds and volatiles were observed throughout processing/fermentation of *Kapi*. According to overall results, shrimp *A. vulgaris* could be used as alternative raw material for making *Kapi*. Several

changes of *Kapi* components took place throughout all processes, and mainly occurred during fermentation period. Those changes played a role in the characteristic of resulting *Kapi,* especially taste or flavor. Moreover, *Kapi* was shown as the higher potential source of natural antioxidants, compared to fresh shrimp.

9.7 References

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CHAPTER 10

MICROBIOLOGICAL AND CHEMICAL CHANGES OF SHRIMP *ACETES VULGARIS* DURING *KAPI* PRODUCTION

10.1 Abstract

Microbiological and chemical changes in shrimp *Acetes vulgaris* during production of *Kapi* (salted shrimp paste of Thailand) including salting, drying and fermentation were monitored. Moisture content of samples decreased rapidly after salting and drying steps. The lower water activity was found in the final product (0.694). The pH decreased within the first 10 days of fermentation and continuously increased as fermentation progressed. Protein underwent degradation throughout *Kapi* production as indicated by increasing TCA-soluble peptides and degree of hydrolysis. The increases in peroxide value as well as thiobarbituric acid reactive substances value revealed that lipid oxidation occurred throughout all processes. Total viable count, halophilic, proteolytic and lipolytic bacteria counts increased continuously during *Kapi* production, while lactic acid bacteria count slightly decreased at the final stage of fermentation. Thus, proteolysis and lipolysis took place throughout *Kapi* production, and contributed to the characteristics of finished product. These changes were governed by both endogenous and microbial enzymes.

10.2 Introduction

Kapi, a purplish gray or dark grayish brown salted shrimp paste of Thailand, has been widely used as a condiment in several Thai foods (Pongsetkul *et al.,* 2014). *Kapi* is known with different names in each region, e.g. *Terasi-udang* in Indonesia, *Belacan* in Malaysia, *Bagoong-alamang* in the Philippines, *Mam ruoc* in Vietnam, etc. (Hajeb and Jinap, 2015). Although those products were different in term of raw material, the ratio of salt used, fermentation process and time, they can provide a salty and umami taste to cuisines. Different shrimp are used for *Kapi* production in various regions. Stocks of krill (*Mesopodopsis orientalis*), a traditional raw material for *Kapi* production, have decreased by 3% per year since 1990 (Meland and Willassen,

2007). Other raw materials for *Kapi* production have been researched. *Acetes vulgaris* is one of small shrimp found in the southern part of Thailand throughout the years (Pongsetkul *et al.,* 2015). Our previous study revealed that *Kapi* prepared from *A. vulgaris* had comparable sensory properties to commercial products (Pongsetkul *et al.,* 2015). For *Kapi* production, there are 2 major ingredients including shrimp and salt. The ratio between shrimp and salt varies from 1:6 to 1:2 (w/w) (Hajeb and Jinap, 2015). After salting overnight, salted shrimp is dried with sunlight, ground into a fine paste and fermented under anaerobic condition at ambient temperature (28-30°C) up to 30 days or until the typical aroma is formed (Faithong and Benjakul, 2012). Salting and drying are two ancient practices used for food preservation and yield a particular flavor (Rodrigo *et al.,* 1998). During fermentation, microbiological and biochemical change occur. Proteolysis is an essential biochemical reaction occurring during the fermentation of *Kapi*. It is induced by endogenous proteases in shrimp as well as those produced by halophilic bacteria. Proteolysis affects both texture and flavor of fermented products by inducing the formation of low molecular weight compounds, e.g. peptides, amino acids, aldehydes, organic acids and amines (Mizutani *et al.,* 1987). Lipolysis during fermentation is also important since free fatty acids (FFA) released undergo oxidation, responsible for aroma development (Lizaso *et al.,* 1999). Oxidation products include aldehydes and ketones (Takeungwongtrakul and Benjakul, 2013) and more likely contribute to the development of typical taste and flavor of fermented product (Lizaso *et al.,* 1999). Furthermore, Amano (1962) revealed that growth of anaerobes in high salt environment contributes to the typical flavor of fermented products. Initial microflora, in both the raw materials and salt, have the influence on the numbers of microorganisms in final product, especially during the early stages of fermentation (Rodrigo *et al.,* 1998). Microaerophilic or anaerobic conditions with high salt during *Kapi* fermentation could favor the proliferation of some bacteria, leading to different flavor or odor in the finished products. However, a little information concerning chemical and microbiological changes during *Kapi* processing/fermentation exists.

10.3 Objective

To monitor the changes in chemical compositions and microbial populations of shrimp *A. vulgaris* during *Kapi* production.

10.4 Materials and methods

10.4.1 Sample collection and preservation

Shrimp *A. vulgaris* (average body length 15.6±1.4 mm, average wet weight 0.0413±0.0098 g, n=20) were caught off and collected from the Ko-yo area, Songkhla province, Thailand. After capture, shrimp were delivered to the Department of Food Technology, Prince of Songkla University, Hat Yai, Thailand, in ice using a shrimp/ice ratio of 1:2 (w/w) in a polystyrene container, within approximately 2 h.

10.4.2 Preparation of *Kapi*

Upon arrival, shrimp [R] were mixed with solar salt at the ratio of 5:1 (w/w). The mixture was placed in the basket, covered with a cheese cloth and allowed to salting overnight at room temperature (28-32°C) [S]. Subsequently, salted shrimp were drained, mashed, followed by spreading out on fiberglass mats to dry with sunlight until the moisture content reached 35-40% [D]. After drying, samples were compacted into earthen jars and covered with plastic bag tightly (in the close system). The samples were allowed to ferment at room temperature (28-32°C) for 1 month. During fermentation, the samples were taken at day 10 [F1], 20 [F2] and 30 [F3] of fermentation. All samples were subjected to determination of chemical composition and microbial load.

10.4.3 Changes in chemical compositions

10.4.3.1 Moisture content, water activity (A_w) and pH

Moisture content of all samples was measured as per AOAC method (2000) with the analytical No. of 35.1.13. A_w was determined by a water activity analyzer (Thermoconstanter, Novasina, Switzerland). The pH of samples was analyzed

by a pH meter (Sartorius, Gottingen, Germany) following the method of Nirmal and Benjakul (2009).

10.4.3.2 Free amino acid composition

Free amino acid composition of samples was determined according to the method of Minh-Thuy *et al.* (2014). Free amino acids were firstly extracted using 6% (v/v) perchloric acid. The extracts were subsequently neutralized and filtrated as per the method of Minh-Thuy *et al.* (2014). The filtrate was used for amino acid analysis using an amino acid analysis system (Prominence; Shimadzu, Kyoto, Japan) equipped with a column (Shim-pack Amino-Li, 100 mm×6.0 mm i.d.; column temperature, 39.0°C; Shimadzu) and pre-column (Shim-pack ISC-30/S0504 Li, 150 mm×4.0 mm i.d.; Shimadzu). Amino acids were detected using a fluorescence detector (RF-10AXL; Shimadzu). The content was reported in term of mg/g dry weight sample.

10.4.3.3 TCA-soluble peptide content

TCA-soluble peptide content of samples was determined according to the method of Pongsetkul *et al.* (2015). Ground sample (3 g) was homogenized with 27 ml of cold 5% TCA using a homogenizer at a speed of 11,000 rpm for 1 min. The homogenate was stored in ice for 30 min, followed by centrifugation at 5000×*g* for 20 min at 4°C using a refrigerated centrifuge (Model RC-B Plus centrifuge Newtown, CT). Soluble oligopeptide content in the supernatant was measured according to the Lowry method (Lowry *et al.,* 1951) and expressed as mmol tyrosine equivalent/g dry weight sample.

10.4.3.4 Degree of hydrolysis (DH)

DH of all samples was determined following the method of Benjakul and Morrissey (1997). DH was calculated and expressed as the percentage as follows:

$$\text{Degree of hydrolysis (\%)} = \frac{L}{L_{max}} \times 100$$

where L is the amount of free amino group in the product and L_{max} is the total free amino group after acid hydrolysis (6 M HCl at 100°C for 24 h).

10.4.3.5 Peroxide value (PV)

Lipids of sample were extracted using Bilgh and Dyer (1959) method. PV of lipid samples was determined as described by Sae-leaw *et al.* (2013). Cumene hydroperoxide at a concentration range of 0.5-2 ppm was used for standard preparation. PV was calculated and expressed as mg hydroperoxide/kg dry sample.

10.4.3.6 Thiobarbituric acid reactive substances (TBARS)

TBARS value was examined following the method of Nirmal and Benjakul (2009). A standard curve was prepared using malondialdehyde bis (dimethyl acetal) (0-2 ppm). TBARS value was calculated and expressed as mg malondialdehyde (MDA)/kg dry sample.

10.4.4 Changes in microbial load

10.4.4.1 Total viable count

Total viable count was determined using a standard plate count agar containing 10% NaCl (pH 7.5) and the incubation was performed for 3-5 days at 30°C (BAM, 2001). Samples (25 g) were mixed with 225 ml of peptone water containing 10% (w/v) NaCl in a Stomacher 400 Lab Blender (Seward Ltd., Worthing, UK) at high speed for 3 min. Peptone water containing 10% (w/v) NaCl was used for sample dilution. The sample appropriately diluted in serial tenfold steps was used for analysis by the spread plate technique. Microbial load was reported as colony forming units/g dry sample (CFU/g dry sample).

10.4.4.2 Halophilic bacteria count

Halophilic bacteria content was measured using JCM media (Namwong *et al.,* 2009). The medium (1 L) consisted of 5 g of casamino acid, 5 g of yeast extract, 1 g of sodium glutamate, 3 g of trisodium citrate, 20 g of $MgSO_4 \cdot 7H_2O$, 2 g of KCl,

150 g of NaCl, 0.036 g of $FeCl_2 \cdot 4H_2O$, 0.00036 g of $MnCl_2 \cdot 4H_2O$ and 20 g of agar (pH 7.5). Diluted sample (0.1 ml) was applied on the surface of media, spread and incubated for 5-7 days at 30°C.

10.4.4.3 Proteolytic bacteria count

Proteolytic bacteria count was determined using a standard plate count agar having 10% NaCl and 1% (w/v) sodium caseinate (pH 7.5) with the incubation for 3-5 days at 30°C (Tanasupawat *et al.,* 2011). Proteolytic bacteria, which showed clear zone around colonies on plate, were counted.

10.4.4.4 Lipolytic bacteria count

Lipolytic bacteria count was determined using a standard plate count agar including 10% NaCl and 1% (w/v) tributyrin (pH 7.5). The incubation for 3-5 days at 30°C was performed (Chappe *et al.,* 1994). Lipolytic bacteria showing clear zone around colonies on plate were counted.

10.4.4.5 Lactic acid bacteria (LAB) count

LAB count was determined using De Man, Rogosa, and Sharpe (MRS) agar containing 10% NaCl following the method of Tanasupawat *et al.* (2011). Aliquot of 0.1 ml of appropriately decimally diluted samples was introduced on MRS agar containing $CaCO_3$ (1%) and 10% NaCl (pH 7.5) and incubated for 3-5 days at 30°C.

10.4.5 Statistical analysis

All experiments were conducted in triplicate using three lots of samples. Statistical analysis was done by using oneway analysis of variance (ANOVA). Mean comparison was conducted using the Duncan’s multiple range test (Steel *et al.,* 1980). Analysis was performed using the SPSS package (Version 10.0) (SPSS for windows, SPSS Inc., Chicago, IL, USA).

10.5 Results and discussion

10.5.1 Changes in chemical compositions of shrimp during *Kapi* production

10.5.1.1 Changes in moisture content, A_w and pH

Moisture content of shrimp during *Kapi* production was monitored (Figure 27A). Fresh shrimp had the moisture content of 83.53%. Moisture content of shrimp after being salted overnight was rapidly lowered to 66.03% ($p < 0.05$). During salting, salt was able to penetrate into the shrimp meat, whereas water was removed from shrimp via the osmotic pressure. This led to the decrease in moisture content (Chaijan, 2011). During the first 10 h of fish flesh salting, water content decreased more rapidly during dry salting and the amount of exudate was related with lowered moisture content (Bellagha *et al.,* 2007). Moisture content of salted shrimp sharply decreased after drying with the sunlight and low moisture content (32.43%) was obtained after this process. Moisture content of samples was slightly increased after the first 10 days of fermentation ($p < 0.05$). Partially dried sample more likely absorbed the water vapor from the environment to some degree. However, no difference was observed among samples as fermentation time was extended up to 30 days ($p < 0.05$). Moisture content directly affected the consistency of *Kapi.* In general, consistency of commercial *Kapi* was varied from soft and pasty to dry and hard, depending upon the processes used (Faithong *et al.,* 2010). Moisture content was within the moisture range of commercial *Kapi* produced in Thailand (33.79-52.54%) (Pongsetkul *et al.,* 2014). After 30 days of fermentation, our product had the moisture content of 35.07%. This was associated with texture, because it became slightly dry and hard. Moreover, the result also suggested that salting and drying step for *Kapi* production were the main steps to reduce the moisture content.

Changes in water activity (A_w) of all samples exhibited the similar trends with those of moisture content throughout *Kapi* production processes as depicted in Figure 27B. Decreases in A_w of shrimp during salting and drying were observed ($p < 0.05$) and no changes in A_w were obtained during 30 days of fermentation ($p < 0.05$). A_w of final product (after 30 days of fermentation) was 0.694, corresponding to low moisture content. Food having A_w range of 0.6-0.7 could be classified as an

intermediate moisture food (Fennema, 1996). Since *Kapi* samples had A_w in this range, the growth of food pathogens and spoilage microorganisms could be retarded.

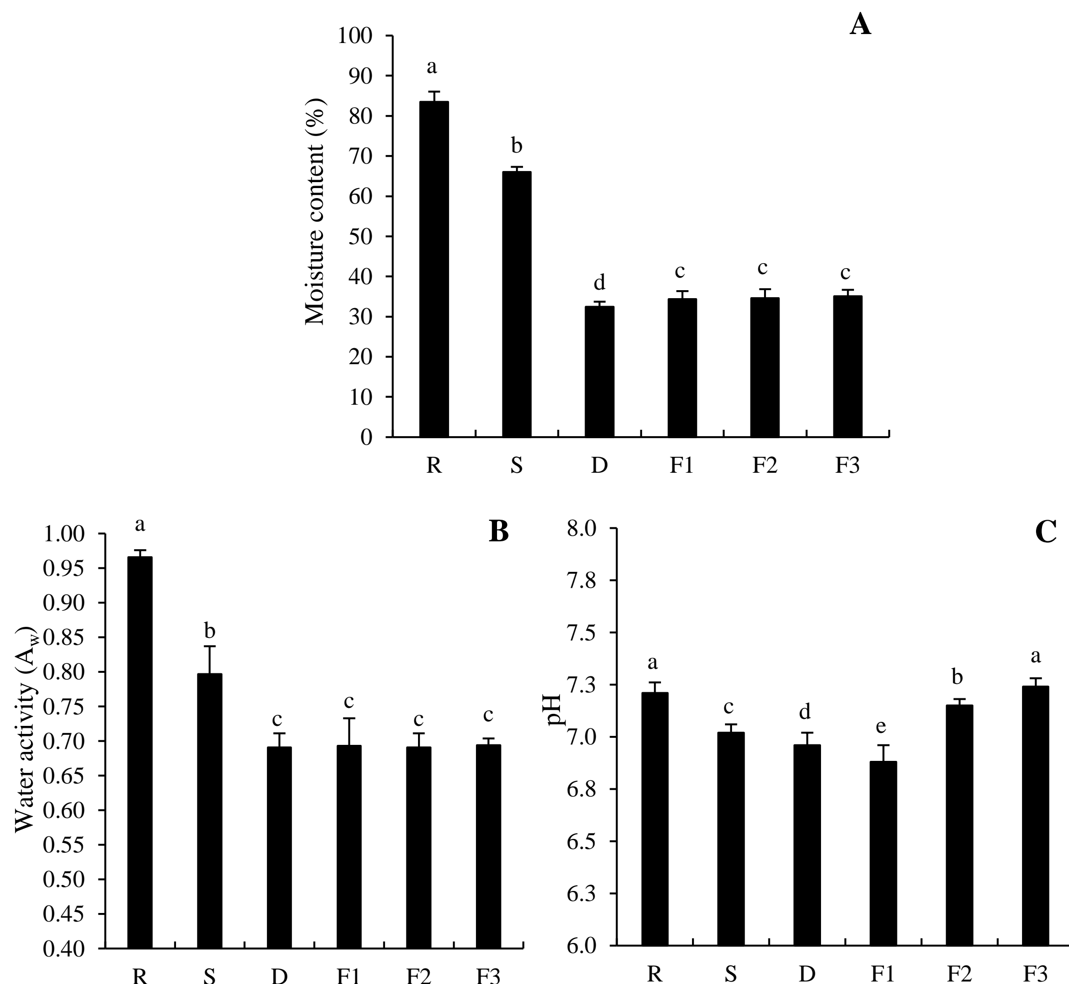

Figure 27. Changes in moisture content (A), water activity (A_w) (B) and pH (C) of shrimp during processing/fermentation of *Kapi*. R: raw material; S: after salting; D: after drying; F1, F2 and F3: after fermentation for 10, 20 and 30 days.

Bars represent the standard deviation from triplicate determinations. Different letters on the bars denote the significant differences ($p < 0.05$).

Changes in the pH of shrimp throughout all processes were found. Fresh shrimp had the pH of 7.21. pH of sample was slightly dropped during salting (7.02) and drying (6.96) ($p < 0.05$) as shown in Figure 27C. Continuous decrease in pH within the first 10 days of fermentation was observed ($p < 0.05$). Some microorganisms, particularly LAB, were able to produce some acids, which could

result in pH lowering. These LAB produced lactic acid, leading to the decrease in pH of samples. However, pH turned to increase when fermentation time was more than 20 days ($p < 0.05$). This was related with the decrease in LAB. Furthermore, the increase of pH during fermentation was plausibly associated with the formation of degradation products or volatile base compounds such as ammonia produced by endogenous or microbial enzymes (Pongsetkul *et al.,* 2015). After 30 days of fermentation, the pH of final product was 7.24.

10.5.1.2 Changes in free amino acid composition

Free amino acid composition of shrimp throughout all processes is given in Table 29. Total free amino acid (TFAA) of fresh shrimp was 44.75 mg/g dry sample. There was no change in TFAA when fresh shrimp were subjected to salting and drying ($p > 0.05$). However, TFAA was continuously increased during fermentation and reached 64.66 mg/g dry sample after the fermentation was performed for 30 days ($p < 0.05$). Change in total essential amino acid (TEAA) was fluctuated during *Kapi* production, but there were no differences between fresh shrimp and final product ($p < 0.05$). On the other hand, total nonessential amino acid (TNEAA) contents were different among samples ($p < 0.05$). The increases in TNEAA during all processes were in agreement with the increases in TFAA. TNEAA reached 49.17 mg/g dry sample after 30 days of fermentation.

Fresh shrimp *A. vulgaris* contained Glu/Gln, Arg, Asp/Asn, Ala, Pro and Gly as the major free amino acids and their total amount was more than 50% of total free amino acids. The result was in agreement with the other shrimp species including *Acetes chinesis* (Chung and Lee, 1976), *Penaeus notialis* (Akintola, 2015), *Penaeus kerathurus* (Zlatanos *et al.,* 2009), etc. It was noted that changes in amount of each amino acid varied during different processes. As processing proceeded, some amino acids, especially Glu/Gln, Asp/Asn increased, while other amino acids, particularly Ile, Arg and Ser decreased. Some amino acids, which were not found in fresh sample, were generated during *Kapi* processing. Those included Trp, Ans, Car, Cit, Cys, etc. These changes in free amino acid during processing might be influenced by endogenous proteases in raw material and microbial proteases proliferated during processes.

Table 29. Changes in free amino acid composition of shrimp during processing/ fermentation of *Kapi*

Amino acid composition[*]	Samples[**]					
	R	S	D	F1	F2	F3
Essential amino acid						
Histidine (His)[***]	0.89±0.01ab	0.93±0.02a	0.85±0.01b	0.54±0.01c	0.55±0.07c	0.59±0.01c
Isoleucine (Ile)	1.56±0.03a	1.30±0.01b	1.32±0.01b	1.21±0.02c	1.18±0.01c	1.18±0.01c
Leucine (Leu)	2.71±0.01c	2.81±0.01c	3.25±0.01b	3.99±0.01a	3.99±0.03a	4.05±0.01a
Lysine (Lys)	2.88±0.03cd	3.01±0.01c	3.03±0.01c	3.95±0.05b	4.26±0.02a	4.38±0.03a
Methionine (Met)	0.72±0.01b	0.75±0.02b	0.72±0.00b	1.12±0.01a	1.09±0.01a	1.16±0.01a
Phenylalanine (Phe)	2.10±0.06a	2.09±0.04a	2.24±0.01a	2.05±0.01a	1.66±0.01b	1.43±0.01b
Threonine (Thr)	1.98±0.02a	1.72±0.04a	1.33±0.01a	0.05±0.00b	0.08±0.01b	0.03±0.00b
Tryptophan (Trp)	ND	ND	ND	ND	0.03±0.00a	0.03±0.01a
Valine (Val)	2.34±0.01b	2.32±0.01b	2.36±0.04b	2.55±0.03a	2.39±0.02ab	2.64±0.03a
Total EAA	15.18±0.06a	14.93±0.04b	15.10±0.03b	15.46±0.05a	15.23±0.06a	15.49±0.03a
Non-essential amino acid						
Alanine (Ala)	3.57±0.02c	3.58±0.04c	4.1±0.04b	5.23±0.06a	5.62±0.04a	5.66±0.02a
Anserine (Ans)	ND	ND	ND	ND	0.03±0.00b	0.06±0.00a
Arginine (Arg)	5.16±0.06a	5.02±0.03a	4.55±0.01b	4.05±0.01c	4.05±0.04c	3.14±0.01d
Asp/Asn[****]	4.13±0.04c	4.55±0.01c	4.53±0.04c	6.69±0.08b	8.04±0.09a	8.51±0.04a
Carnosine (Car)	ND	ND	ND	ND	ND	0.03±0.00
Citrulline (Cit)	ND	ND	ND	ND	0.07±0.00a	0.09±0.01a
Cysteine (Cys)	ND	ND	0.04±0.01c	0.06±0.01b	0.06±0.00b	0.12±0.01a
Glycine (Gly)	3.31±0.02d	3.54±0.01c	3.91±0.05a	3.55±0.01c	3.75±0.03b	3.66±0.02bc
Glu/Gln[*****]	5.42±0.10e	5.35±0.04e	6.02±0.04d	9.11±0.03c	14.15±0.07b	18.18±0.05a
Hydrolysine (Hyl)	ND	ND	ND	ND	ND	0.02±0.00
Hydroxyproline (Hyp)	ND	ND	ND	ND	ND	ND
Ornithine	ND	ND	ND	1.04±0.01b	1.15±0.01b	2.02±0.02a
Proline (Pro)	3.48±0.02c	3.55±0.01c	3.28±0.02c	4.22±0.01b	4.59±0.02ab	5.01±0.01a
Serine (Ser)	2.94±0.05a	2.05±0.02b	2.15±0.01b	1.64±0.02c	0.92±0.00d	0.93±0.01d
Taurine (Tau)	0.02±0.00c	0.02±0.00c	0.02±0.00c	0.07±0.01a	0.05±0.00b	0.05±0.00b
Tyrosine (Tyr)	1.54±0.01d	1.88±0.01b	1.95±0.01ab	2.11±0.01a	1.82±0.01b	1.69±0.03c
Total NEAA	29.57±0.08d	29.54±0.03d	30.58±0.04d	37.77±0.09c	44.3±0.09b	49.17±0.04a
Total amino acid[*]	**44.75±0.08d**	**44.47±0.06d**	**45.68±0.05d**	**53.23±0.09c**	**59.53±0.10b**	**64.66±0.05a**

[*]Amino acid composition was expressed as mg/g sample.
[**]R: raw material; S: after salting; D: after drying; F1, F2 and F3: after fermentation for 10, 20 and 30 days.
[***]Essential amino acid in Children; [****]Asp/Asn: Aspartic acid/Asparagine; [*****]Glu/Gln: Glutamic acid/Glutamine.

Our previous study revealed that proteolytic activity of fresh shrimp was decreased after being salted and dried, but continuously increased during fermentation up to 30 days (Pongsetkul *et al.,* 2016). Those proteases more likely played a profound role in protein degradation, thus contributing to the liberation of free amino acids throughout *Kapi* production. The decrease or fluctuation of amino

acids might be from the utilization of those amino acids by microorganisms for their growth.

Kapi fermented for 30 days contained Glu/Gln and Asp/Asn as the dominant free amino acids. The sharp increases in Glu/Gln and Asp/Asn were observed during fermentation and reached 18.18 and 8.51 mg/g dry sample, respectively ($p < 0.05$) at day 30 of fermentation. This result was in accordance with the high amount of Glu/Gln and Asp/Asn in *belacan* (Hajeb and Jinap, 2015), *terasi* (Mizutani *et al.,* 1987), *seinsanga-pi* (Tyn, 1983) and *bagoong-alamang* (Peralta *et al.,* 2008), shrimp pastes of Malaysia, Indonesia, Myanmar and the Philippines, respectively. In general, the taste and odor of salted shrimp paste were governed by amino acids (Hajeb and Jinap, 2015). Some amino acids known as sweet compounds for fermented foods included Lys, Pro, Ala (Chung and Lee, 1976), Asp (Kim *et al.,* 2005), Gly, Ser and Thr (Liu, 1989). Glu/Gln were associated with meaty or umami taste as reported by Chung and Lee (1976).

Based on the result, free amino acid compositions of final *Kapi* product was similar to those found in raw material used, inferring that different raw material with different amino acids more likely yielded *Kapi* with various tastes or flavors. However, those amino acids could be altered during processing, especially during fermentation.

10.5.1.3 Changes in TCA-soluble peptide contents and DH

As shown in Figure 28A, fresh shrimp contained TCA-soluble peptides of 80.62 mmol/g dry weight sample. Pongsetkul *et al.* (2015) reported that fresh *A. vulgaris* contain some TCA-soluble peptides, which could be associated with rapid degradation after death, particularly during transportation. The initial level of TCA-soluble peptides might represented endogenous oligopeptides in sample and the degradation products generated during post-harvest handling (Benjakul and Morrissey, 1997). The increase in the contents of TCA-soluble peptides during *Kapi* production suggested that the degradation mediated by either endogenous or microbial proteases in the presence of high salt still took place. Slight increase in TCA-soluble peptide contents was found after drying process, while the higher rate of increase was noticeable when dried shrimp were fermented at room temperature ($p < 0.05$). After 30

days of fermentation, TCA-soluble peptide content was 288.96 mmol/g dry weight sample, which was 3.58-fold higher than that obtained in raw material. This suggested the continuous proteolysis during fermentation. Increasing TCA-soluble peptides throughout *Kapi* production process was coincidental with the increase in degree of hydrolysis (DH). DH of *Kapi* samples increased from 3.81% in raw material to 21.12% after 30 days of fermentation as shown in Figure 28B. Rapid increase in DH was also found during fermentation process. With increasing fermentation time, short chain peptides were more produced, affecting the characteristics, bioactivities as well as acceptability of *Kapi*.

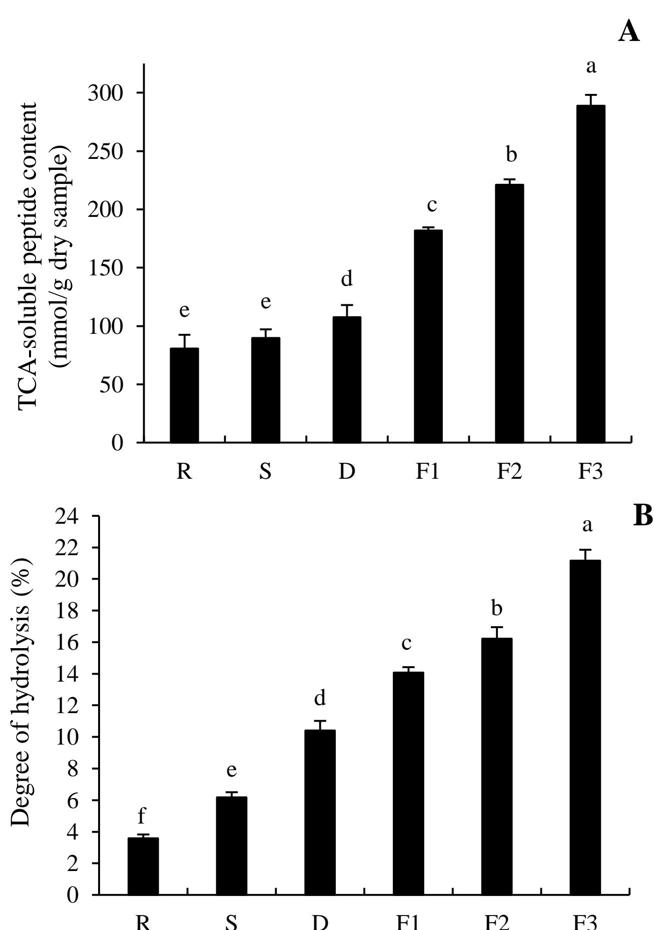

Figure 28. Changes in TCA-soluble peptide content (A) and degree of hydrolysis (B) of shrimp during processing/fermentation of *Kapi*. R: raw material; S: after salting; D: after drying; F1, F2 and F3: after fermentation for 10, 20 and 30 days.

Bars represent the standard deviation from triplicate determinations. Different letters on the bars denote the significant differences ($p < 0.05$).

10.5.1.4 Changes in lipid oxidation products

Lipid oxidation of shrimp during *Kapi* production expressed as PV and TBARS values was monitored (Figure 29A and 29B). PV of fresh shrimp was 0.08 mg hydroperoxide/kg dry sample, suggesting that lipid oxidation occurred after harvest or during transportation in ice. There were no differences in PV between fresh sample and that after salting ($p < 0.05$). However, PV increased after drying with sunlight and continuously increased during fermentation up to 20 days. Subsequently, a decrease in PV was noticeable at day 30 of fermentation ($p < 0.05$). The increase in PV during processing and the first period of fermentation more likely resulted from the formation of hydroperoxide, a primary lipid oxidation products. Lipid hydroperoxides are formed by various pathways. Those include the reaction of singlet oxygen with unsaturated fatty acids or the lipoxygenase-catalyzed oxidation of PUFA (Sae-leaw *et al.,* 2013). The decrease in PV after 30 days of fermentation was probably due to the decomposition of hydroperoxide to the secondary oxidation products. Shrimp oil was rich in polyunsaturated fatty acids (Takeungwongtrakul and Benjakul, 2013). The result indicated that lipid oxidation occurred during *Kapi* production, probably owing to the high content of polyunsaturated fatty acids. Lipid oxidation products could affect odor and flavor characteristics of *Kapi*.

TBARS values of shrimp during *Kapi* production is shown in Figure 29B. TBARS value of 0.23 mg MDA/kg dry sample was obtained in fresh shrimp before salting process, suggesting the presence of lipid oxidation products. No changes in TBARS value were noticeable after being salted ($p < 0.05$). Thereafter, a continuous increase in TBARS value during drying and fermentation was found ($p < 0.05$). TBARS value is the good index of concentration of relatively polar secondary reaction products, especially aldehydes, which are considered to be responsible for the off-flavors in meat (Cagdas and Kumcuoglu, 2015). As fermentation time increased, lipid oxidation proceeded in sample as evidenced by the increases in TBARS value. At the end of process (30 days of fermentation), TBARS value was increased to 0.94 mg MDA/kg dry sample (fourfold higher than that of fresh sample). As fermentation time increased, shrimp matrix was loosened, caused by autolysis or microbial action. Thus,

lipids bound with matrix were more released and exposed to oxidation, as indicated by the increases in both PV and TBARS values.

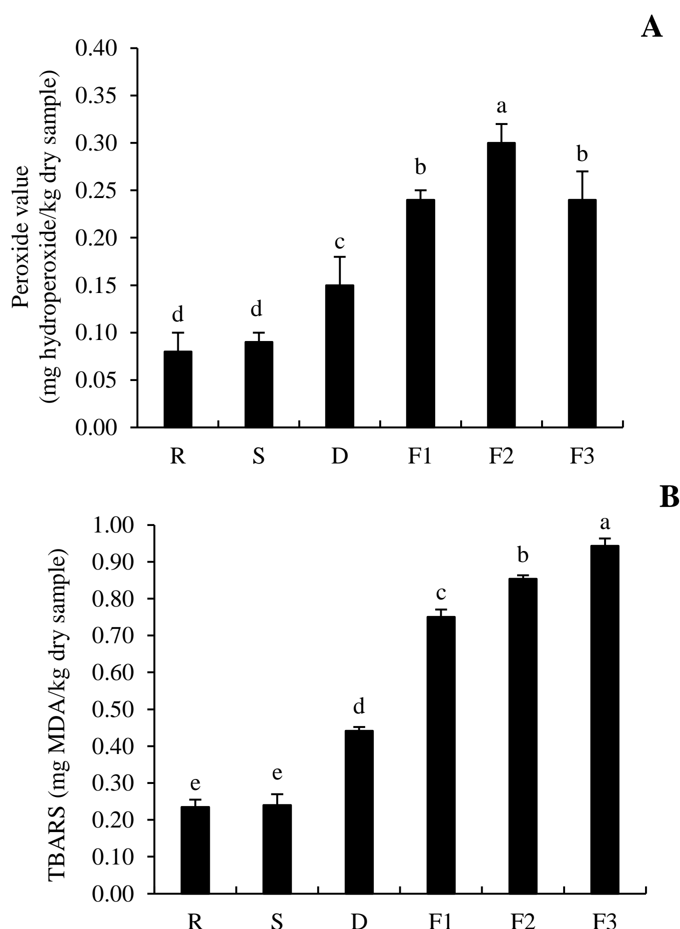

Figure 29. Changes in peroxide values (A) and TBARS values (B) of shrimp during processing/fermentation of *Kapi*. R: raw material; S: after salting; D: after drying; F1, F2 and F3: after fermentation for 10, 20 and 30 days.

Bars represent the standard deviation from triplicate determinations. Different letters on the bars denote the significant differences ($p < 0.05$).

10.5.2 Changes in microbial populations of shrimp during *Kapi* production

Initial total viable count (TVC) of shrimp used as raw material was 3.50 log CFU/g dry sample and slightly decreased after salting and drying process ($p < 0.05$) (Figure 30A). Nevertheless, TVC continuously increased during fermentation at

room temperature ($p < 0.05$) and reached 5.84 log CFU/g dry sample after 30 days of fermentation. Microorganisms found in fresh shrimp could be contaminated during handling or washing using seawater. In addition, sorting to remove foreign materials is commonly carried out, which is typically done by hand. This might introduce microorganisms to shrimp. The presence of microorganisms during fermentation contributes to the degradation of proteins and development of flavor and aroma (Majumdar *et al.,* 2015). For the production of *Kapi,* salt plays a key role in preservation by inhibiting the growth of spoilage and pathogenic microorganisms. Phithakpol (1993) reported that putrefactive microorganisms are inhibited by salt at level higher than 6-8%. Salting and drying processes led to the lower moisture content and water activity (A_w) of shrimp as shown in Figure 27A and 27B, respectively. These processing conditions could be both eradicate and select microorganisms. It was found that slight reduction of TVC after drying process was observed ($p < 0.05$). In contrast, TVC rapidly increased as fermentation increased ($p < 0.05$), especially within the first 10 days of fermentation. The selected strains might grow well under fermentation condition. The increase in microbial population was correlated well with the rapid increase in TCA-soluble peptides and DH (Figure 28) during fermentation. Latorre-Moratalla *et al.* (2011) revealed that free amino acids and other soluble non-nitrogenous substances derived from proteolysis could be considered as available nutrients for microorganisms. In general, the increase in TVC was in accordance with increasing free amino acids throughout the process (Table 29). Moreover, decomposition and oxidation of lipids during *Kapi* fermentation, as indicated by PV and TBARS (Figure 29), also occurred as induced by the increase in these microorganisms.

An increase in the number of halophilic or halo-tolerant bacteria was observed after salting and drying and also continuously increased during fermentation up to 30 days as shown in Figure 30B. The high salt environment and microaerophilic or anaerobic conditions during *Kapi* processing and fermentation more likely resulted in the proliferation of halophilic bacteria. The increased halophilic bacteria count during *Kapi* production coincided well with the increasing TVC. This result suggested that dominant microorganisms in *Kapi* were halophilic bacteria. Chuon *et al. (*2014)

revealed that halophilic and halotolerant bacteria genera, *Staphylococcus* and *Tetragenococcus*, were predominant microorganisms found in *Kapi* from Cambodia.

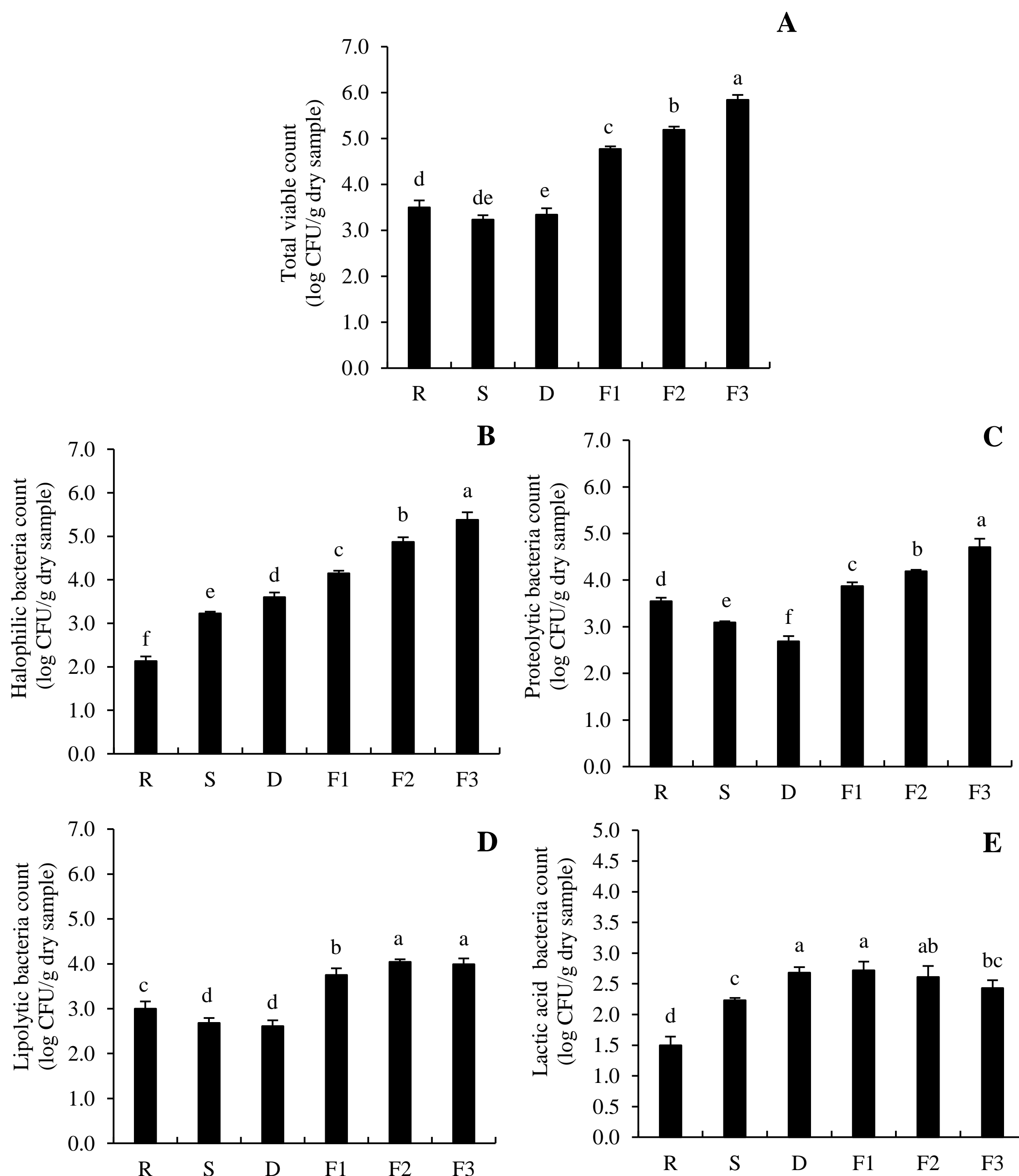

Figure 30. Changes in total viable count (A), halophilic bacteria (B), proteolytic bacteria (C), lipolytic bacteria (D) and lactic acid bacteria (E) of shrimp during production/fermentation of *Kapi*. R: raw material; S: after salting; D: after drying; F1, F2 and F3: after fermentation for 10, 20 and 30 days.

Bars represent the standard deviation from triplicate determinations. Different letters on the bars denote the significant differences ($p < 0.05$).

However, Kobayashi *et al.* (2003) reported that *Bacillus licheniformis* and *Bacillus sphaericus* were found in Indonesian fermented shrimp (*Terasi*). Moreover, halophilic bacteria found in *Kapi* showing proteolytic activity included *Oceanobacillus kapialis* (Namwong *et al.,* 2009), *Lentibacillus kapialis* (Pakdeeto *et al.,* 2007) and *Virgibacillus halodenitrificans* (Tanasupawat *et al.,* 2011), etc.

The increase in proteolytic bacteria count during fermentation was also observed in the present study (Figure 30C). Furthermore, as shown in Figure 30D, lipolytic bacteria count also increased during fermentation of *Kapi*. Halophilic bacteria might contain lipolytic enzymes. This resulted in the release of free fatty acids, which were susceptible to oxidation as indicated by the increases in PV and TBARS values (Figure 29). The initial proteolytic and lipolytic bacteria counts in fresh shrimp were 3.55 and 3.00 log CFU/g dry sample, respectively. Both types of bacteria showed similar changes throughout *Kapi* production. Both proteolytic and lipolytic bacteria counts of fresh shrimp were slightly decreased after drying process ($p < 0.05$). The presence of high salt as well as the lowered water activity after drying in this product could retard the growth of proteolytic or lipolytic microorganisms. However, both proteolytic and lipolytic bacteria counts increased during fermentation and reached the highest level at day 30 of fermentation ($p < 0.05$).

Figure 30E shows the changes in lactic acid bacteria (LAB) count of shrimp during *Kapi* production. The initial LAB count was quite low (about 1.50 log CFU/g dry sample). LAB count increased continuously until drying process was complete ($p < 0.05$). No further change in LAB count occurred during fermentation up to 20 days ($p < 0.05$). Conversely, slight decrease was found as fermentation was performed for 30 days ($p < 0.05$). In general, LAB species, especially belonging to *Tetragenococcus*, are known to be dominant microorganisms in many high-salt-containing fermented foods (Chuon *et al.,* 2014). LAB plays a profound role in flavor of those products due to their fermentation of carbohydrates. The limited increase in LAB count after salting, drying as well as the first 10 days of fermentation was observed with the slight decrease in pH (Figure 27C). No further growth of LAB was noticeable after 20 days of fermentation, while pH was slightly increased. It was suggested that LAB were not predominant or played an important role in *Kapi* fermentation with the extended fermentation time. Also, proteins in *Kapi* plausibly

exhibited buffering capacity, thereby preventing the pH lowering. Furthermore, *Kapi* contained low amount of carbohydrate as reported by Pongsetkul *et al.* (2014) and Faithong *et al.* (2010). This could result in the reduction of LAB during fermentation because of insufficient substrates for their growth.

Overall, halophilic bacteria were the dominant microorganisms, which could grow and produce some proteolytic or lipolytic enzymes, which play a profound role in degradation/decomposition of proteins and lipids in shrimp throughout the process. Those degradation products might contribute to the typical characteristics of final products, especially flavor or taste. As reported by Phithakpol (1993), the stronger aroma was obtained in *Kapi* with increasing fermentation time. Therefore, the development of flavor or taste of *Kapi* could be related with the increasing microbial load during *Kapi* fermentation.

10.6 Conclusion

Chemical compositions and microbial populations of shrimp underwent changes throughout the processes used for *Kapi* production. Salting and drying were mainly implemented to lower moisture content and A_w, in order to prolong the shelf-life of this product. Protein degradation and lipid oxidation occurred throughout *Kapi* production. For microorganisms, TVC increased continuously and halophilic bacteria were prevalent. Proteolytic and lipolytic bacteria were also increased throughout all processes. Therefore, proteolysis and lipolysis, mediated by both endogenous and microbial enzymes, were involved throughout the process and contributed to the characteristics of finished product. Isolation and identification of these bacteria isolated from *Kapi* production will be further studied. The use of the selected isolates as starter culture for production of *Kapi* with desired quality needs to be investigated.

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CHAPTER 11

CHANGES IN LIPIDS OF SHRIMP *ACETES VULGARIS* DURING SALTING AND FERMENTATION

11.1 Abstract

Changes in lipids of shrimp *Acetes vulgaris* during production of *Kapi* (salted shrimp paste of Thailand) were investigated. Shrimp had lipid content of 4.21% (dry weight basis). Lipids mainly consisted of triacylglycerol (TAG) (28.03 g/100 g) and sterol (ST) (24.03 g/100 g). Lipid content decreased during salting, drying and the first period of fermentation ($p < 0.05$). Both TAG and ST gradually decreased, while free fatty acid (FFA) increased and became dominant in the final product (49.29%). Phospholipids including phosphatidylethanolamine (PE) (4.80 g/100 g) and phosphatidylcholine (PC) (5.67 g/100 g) were also found in fresh shrimp and still remained in the final product. Palmitic acid (C16:0) was the major fatty acid in shrimp (27.95%). Eicosapentaenoic acid (EPA) (C20:5(n-3)) and docosahexaenoic acid (DHA) (C22:6(n-3)) were found at high content (19.03%). When different fractions were isolated from crude lipids, varying fatty acid profiles were obtained. TAG and FFA fractions mainly consisted of saturated and monounsaturated fatty acids. Polyunsaturated fatty acids were accumulated in PE and PC fractions. Peroxide value and TBARS value increased during *Kapi* production, suggesting that lipid oxidation took place. Overall, lipolysis and oxidation of shrimp lipids occurred throughout the processes, thereby affecting lipid constituents. Those changes might partially contribute to the *Kapi* characteristics, especially flavor.

11.2 Introduction

Shrimp and shrimp-derived foods are generally known as a good source of polyunsaturated fatty acids (PUFA) such as docosahexaenoic acid (DHA), eicosapentaenoic acid (EPA), and arachidonic acid (AA) (Uauy *et al.,* 2000). These PUFA have many beneficial effects on human health, e.g., lowering the incidence of cardiovascular diseases and sudden cardiac death (Villa *et al.,* 2002), being essential

for the formation of neuron synapses in the fetal brain (Jensen, 2006), and suppressing the increase of plasma cholesterol (Uauy *et al.,* 2000). However, the synthesis of PUFA from human body is inefficient, especially in infants. To satisfy the nutritional requirements, PUFA must be obtained from the diet, or produced in vivo from diet-derived n-3 fatty acid precursors such as α-linolenic acid (Field *et al.,* 2001).

Kapi is the typical traditional salted shrimp paste of Thailand and is consumed as condiment for a variety of Thai Foods. *Kapi* is normally made of small shrimp/krill and solar salt at the ratio of 5:1. The mixture of shrimp/krill and salt is allowed to stand overnight, followed by drying with sunlight and fermentation at the room temperature, respectively. Fermentation of *Kapi* generally takes at least 1 month or until the typical flavor is obtained (Pongsetkul *et al.,* 2014). The drying and fermentation not only produce the typical sensory characteristics and render microbiological stability, but also affect the composition and nutritional value. Since the stocks of krill (*Mesopodopsis orientalis*), a traditional raw material, have dropped by 3% per year over the last decade (Meland and Willassen, 2007), small shrimp *Acetes vulgaris*, by-catch from commercial fishing, became the alternative raw material for *Kapi* manufacturing, owing to its high availability throughout the years and low price, especially in the southern part of Thailand. Our previous study revealed that *Kapi* prepared from *A. vulgaris* had comparable sensory properties to commercial products made from krill (Pongsetkul *et al.,* 2016).

Lipid is an important constituent, determining both functionality and sensory properties of fermented products (Visessanguan *et al.,* 2006). Lipids, mainly triacylglycerols (TAG) located in muscle and adipose tissues and phospholipids (PL) found in membrane of muscular cells, are largely involved in sensory attributes of several food products. During processing, lipolysis and oxidation can take place, thus yielding numerous volatile compounds (Zhou and Zhao, 2007). Lipolysis induced by both endogenous and microbial enzymes occurs in meat and fish during fermentation (Adams, 2010). Lipolysis plays a major role in the fermented foods, in which free fatty acids (FFA) are liberated and further undergo oxidation (Lizaso *et al.,* 1999). Oxidation products such as aldehydes and ketones are responsible for the development of flavor or aroma (Takeungwongtrakul and Benjakul, 2013). Shrimp, especially hepatopancreas located in cephalothorax, is rich in lipids especially PUFA (Takeungwongtrakul *et al.,*

2012). Those lipids, which prone to lipolysis and lipid oxidation, could determine flavor characteristics of *Kapi*. However, no information regarding lipids from shrimp *A. vulgaris* and their changes during *Kapi* production exists.

11.3 Objective

To characterize lipids from shrimp *A. vulgaris* and to monitor their changes during processing of *Kapi.*

11.4 Materials and methods

11.4.1 Sample collection

Shrimp *A. vulgaris* (average body length: 14.3±1.2mm, average wet weight: 0.0427±0.0068 g, n=20) were collected from the offshore area of Ko-yo, Songkhla province, Thailand. Shrimp were immediately placed into the polyethylene bag. The bag was imbedded in a polystyrene box containing ice with a shrimp/ice ratio of 1:2 (w/w) and transported to the laboratory within approximately 2 h.

11.4.2 Preparation of *Kapi*

Upon arrival, shrimp [R] were washed with 3% NaCl solution and transferred into the basket. Solar salt was added at the shrimp/salt ratio of 5:1 (w/w). The mixture was mixed well and allowed to stand at room temperature (28-30°C) overnight [S]. After salting, drained shrimp were pounded until uniformity was obtained. The ground sample was then dried with sunlight by spreading out on fiberglass mats. The drying was continued until the moisture contents of dried shrimp were in range of 35-40% [D]. Thereafter, dried shrimp were impacted into the earthen jars, covered tightly with the plastic bag (close system), and allowed to ferment at room temperature (28-30°C) for 30 days. During fermentation, samples were taken at day 10 [F1], 20 [F2], and 30 [F3] of fermentation. The samples during *Kapi* production were subjected to lipid analysis, in comparison with fresh shrimp. Lipid oxidation products were also examined.

11.4.3 Extraction and characterization of lipids

11.4.3.1 Lipid extraction

Lipids were extracted by the Bligh and Dyer method (Bligh and Dyer, 1959). Lipid content was calculated and expressed as the percentage (dry weight basis). Lipid samples were placed in a vial, flushed with nitrogen gas, sealed tightly, and kept at -40°C until analyses.

11.4.3.2 Analyses of lipids

- Lipid classes

Lipid constituents were separated and classified as per the method of Osako *et al.* (2006) Crude lipids (100 mg) were sequentially separated into different classes on silicic acid chromatographic columns (Kieselgel60, 70-230 mesh, Merck KGaA Co., Ltd, Darmstadt, Germany) using various eluents. Each fraction was quantified by gravimetric method. The first eluate (chloroform/n-hexane, 2:3, vol/vol) was collected as the steryl ester (SE), which contains a small amount of wax ester (WE). The subsequent elution was carried out as follows: chloroform eluting the TAG; chloroform/ether (35:1, vol/vol) eluting the sterols (ST); chloroform/ether (9:1, vol/vol) eluting the DG; chloroform/methanol (9:1, vol/vol) eluting the FFA; chloroform/ methanol (1:1, vol/vol) eluting the PE; chloroform/methanol (1:5, vol/vol) eluting the other minor phospholipids [OPL]; and chloroform/methanol (1:20, vol/vol) eluting the PC. Individual fraction was also identified for lipid classes by comparison with standards using TLC (thickness: 0.25 mm, Kieselgel 60, Merck, Darmstadt, Germany). Each fractions were applied on TLC plates, and the separation was carried out using a mixture of (i) chloroform and hexane (50:50, v/v) for SE and ST; (ii) chloroform for TAG, DG, and MG; and (iii) chloroform and methanol (75:25, v/v) for PE, OPL, and PC as mobile phases.

All fractions were evaporated, flushed with nitrogen gas until dried at room temperature. The samples were stored at -80°C in a small amount of chloroform until analyzed.

- Fatty acid (FA) profile

Total lipids (TL) as well as the selected lipid fractions including TAG, FFA, PE, and PC fractions, were converted into fatty acid metyl esters (FAMEs) as per the method of Saito *et al.* (2005). Lipids were directly trans-esterified with boiling methanol containing 1% hydrochloric acid under the reflux for 1.5 h. Subsequently, FAMEs were purified using silica gel column chromatography by elution with a mixture of n-hexane and ether (10/1, v/v).

Analysis of FAME was performed using a gas chromatography (GC-17A, Shimadzu Seisakusho Co., Ltd, Kyoto, Japan) equipped with a 30m±0.25 mm; 0.25 mm fused-silica capillary column (Omegawax1-250, Supelco Japan Co., Ltd., Tokyo, Japan). The spit ratio was 10:1. The column was maintained at 205°C, while the injection port and detector temperature was set at 290°C. Helium gas was used as carrier with a flow rate of 0.8 ml/min. Quantification of individual fatty acid was performed using Shimadzu Model C-R7A electronic integrator (Shimadzu Seisakusho Co., Ltd., Kyoto, Japan). The amount of individual fatty acid in the sample was expressed as relative percentage of peak area to total peak area. Individual peaks appearing in GC-FID chromatogram were further identified by GC-MS after 4,4-dimethyloxazoline derivatization.

To analyze FAMEs by using GC-MS, DMOX derivatives were prepared as per the method of Saito *et al.* (2005). Excess amount of 2-amino-2-methyl-propanol was added to small amount of FAME under nitrogen atmosphere. The mixture was heated at 180°C for 18 h. After cooling, the reaction mixture was poured onto saturated brine, and extracted by *n*-hexane in triplicate. The extract was washed with saturated brine, before drying with anhydrous sodium sulfate. The solvent was then removed from the extract under reduced pressure condition. The samples were dissolved again in *n*-hexane for analysis by GC-MS.

GC-MS QP 2010 (Shimadzu Seisakusho Co., Ltd.) was used for analysis of the DMOX derivatives. The same capillary column as described above was used with 1:76 split ratio. The temperatures of the injector and the column were held at 240 and 210°C, respectively. MS spectra were recorded at 70 eV ionization energy. Helium gas was used as the carrier gas with a flow rate of 0.7 ml/min.

FAMEs were identified using (i) marine lipid methyl esters as standards (Supelco37 Component FAME Mix, Supelco Japan Co., Ltd.) and also by (ii) comparison of mass spectral data obtained from GC-MS.

11.4.3.3 Determination of lipid oxidation products

- Peroxide value (PV)

PV was measured following the method of Richards and Hultin (2002). Ground sample (1 g) was homogenized at a speed of 13,500 rpm for 2 min in 11ml of chloroform/methanol mixture (2:1, v/v). Homogenate was then filtered using a Whatman No. 1 filter paper. Two milliliters of 0.5% NaCl were then added to 7ml of the filtrate. The mixture was vortexed at a moderate speed for 30 s and then centrifuged at 3000×*g* for 3 min to separate the sample into two phases. Two milliliters of cold chloroform/methanol mixture (2:1) were added to 3ml of the lower phase. Twenty-five microliters of ammonium thiocyanate and 25 ml of iron (II) chloride were added to the mixture. Reaction mixture was allowed to stand for 20 min at room temperature. The absorbance was read at 500 nm. A standard curve was prepared using cumene hydroperoxide at the concentration range of 0-2 ppm. PV was reported as mg hydroperoxide/kg dry sample.

- Thiobarbituric acid reactive substances (TBARS)

TBARS value was determined as per the method of Buege and Aust (1978). Ground sample (1 g) was mixed with 9 ml of 0.25 N HCl solution containing 0.375% TBA and 15% TCA. The mixture was heated in boiling water for 10 min, followed by cooling with the running water.The mixture was centrifuged at 4000×*g* for 20 min (MIKRO20, Hettich Zentrifugan, Germany). The supernatant was collected and the absorbance was read at 532 nm using a UV-160 spectrophotometer (Shimadzu, Kyoto, Japan). A standard curve was prepared using malonaldehyde bis (dimethyl acetal) at concentrations ranging from 0 to 2 ppm. TBARS value was expressed as mg malonaldehyde (MDA)/kg dry sample.

11.4.4 Statistical analysis

Experiments were run in triplicate. The data were subjected to analysis of variance (ANOVA). Comparison of means was carried out by the Duncan's multiple range test. For pair comparison, the t-test were used (Steel *et al.,* 1980). Statistical analysis was performed using the Statistical Package for Social Science (SPSS 11.0 for windows, SPSS Inc., Chicago, IL, USA).

11.5 Results and discussion

11.5.1 Change in lipid content of shrimp during *Kapi* production

Lipid contents of fresh shrimp and those collected during *Kapi* production are shown in Table 30. Fresh shrimp had lipid content of 4.21% (dry weight basis). Generally, lipid content decreased slightly after salting and drying ($p < 0.05$). This was more likely due to the presence of salt in the samples, causing the dilution effect of lipids in salted shrimp. Salt content of shrimp was increased from 0.37% in fresh shrimp to 22.17% in final shrimp paste (% wet weight basis) (Pongsetkul *et al.,* 2015). Additionally, tissues might be disrupted, thus liberating lipids into free form. As a result, the loss in lipids along with the drip from shrimp might occur, particularly during draining after being salted. Decrease in lipid content within the first 10 days of fermentation was also observed ($p < 0.05$). The decrease in lipids was plausibly associated with the utilization of those lipids by microorganisms in the sample. Nevertheless, lipid content remained constant up to 30 day of fermentation ($p > 0.05$). Lipid content of the final *Kapi* was 3.41% (dry weight basis). The decrease in lipid content during processing was also observed in several fermented products such as fermented mackerel (*narezushi*) (Itou *et al.,* 2006) and fermented crab (*ogiri-ssiko*) (Achi *et al.,* 2007).

Table 30. Lipid content of shrimp *A. vulgaris* during *Kapi* production

Samples	Lipid content (% dry weight basis)
Raw material [R]	4.21±0.10[a]
Shrimp after salting [S]	3.92±0.23[b]
Shrimp after drying [D]	3.67±0.32[c]
10 days of fermentation [F1]	3.43±0.17[d]
20 days of fermentation [F2]	3.35±0.22[d]
30 days of fermentation [F3]	3.41±0.10[d]

Mean ± SD (n=3). Different lowercase superscripts in the same column indicate the significant difference ($p < 0.05$).

11.5.2 Change in lipid compositions of shrimp during *Kapi* production

11.5.2.1 Lipid classes

Lipid classes of fresh shrimp and those obtained during *Kapi* production are shown in Table 31. Lipids from fresh shrimp had TAG and ST as the major constituents, accounting for more than 50% of total lipids. SE, DG, and FFA were found as the minor components in shrimp lipids. Polar lipids including PE and PC were also found. High levels of the glycerol derivatives (TAG, PE, and PC) are the typical characteristic of lipids from several crustaceans including Antarctic krill *Euphausia superba* (Ju and Harvey, 2004; Albessard *et al.,* 2001), *Euphausia crystallorophias* (Ju and Harvey, 2004), Northern krill (*Meganyctiphanes norvegicaor*) (Kolattukudy, 1994) and Pacific white shrimp (*Litopenaeus vannamei*) (Takeungwongtrakul *et al.,* 2012). It was noted that fresh shrimp *A. vulgaris* contained SE at a level of 5.20 g/100 g. In general, SE, mainly wax ester, has been found in a wide range of marine animals. Wax esters in epipelagic (mid-water) fish are usually associated with the roe and function as an energy reserve for developing fry. Deep-water fish have wax esters in the muscle and other tissues for helping to control buoyancy (Castille and Lawrance, 1989). The amount of wax esters in crustacean was normally lower than that of marine fish (Ravid *et al.,* 1999). Castille and Lawrence (1989) reported that shrimp *Penaeus aztecus Ives* and *Penaeus setiferus* contained some amount of wax esters, in which 95% were stored in the hepatopancreas. Among all lipids, TAG was found as the most abundant lipid classes in fresh *A. vulgaris* (28.03

g/100 g). In general, TAG is the dominant storage lipid in most fish and shellfish (Ju and Harvey, 2004). TAG is located in fat cells along the muscle fibers and in small cytosolic droplets (Ju and Harvey, 2004). Apart from TAG, shrimp *A. vulgaris* also contained ST as the second dominant constituent (24.03 g/100 g). The presence of ST was in agreement with those reported for other shrimp species such as *Penaeus Semisulcatus* (Ravid *et al.,* 1999), *Orconectes limosus* (Jugan and Herp, 1999), and *Penaeus stylirrostris* (Bray *et al.,* 1990). Free sterols are commonly found in developing ovaries, where they contribute to membrane structure and form precursors for hormones, steroids as well as fat-soluble vitamins (Teshima and Kanazawa, 1971). For the crustaceans, the predominant sterol was cholesterol with only minor amounts of other sterols. A high ratio of cholesterol/noncholesterol sterols was observed in crustaceans (Phillips *et al.,* 2012). Normally, crustaceans contain cholesterol at relatively high levels, compared to meats (Idler and Wiseman, 1971). Cholesterol is an essential nutrient for crustaceans since they are incapable of de novo synthesis of the steroid ring (Phillips *et al.,* 2012). In contrast to cholesterol, PLs, TAGs, and DGs are synthesized by crustacean tissues (Muriana *et al.,* 1993). PLs are the crucial constituents, contributing to the structural and physiological integrity of all cell membranes (Zhi *et al.,* 2010). PLs are considered to be principal lipid components of the hemolymph and tissues of crustaceans, except in the hepatopancreas, where it is rich in neutral lipids (particularly TAG). PLs found in crustaceans were mainly PC and PE, accounting for more than 70% of all PLs (Juan *et al.,* 1992). Zhi *et al.* (2010) reported that PLs of Pacific white shrimp *Litopenaeus vannamei* contained 40% PC and 35% PE. Amount of PC and PE of shrimp *Artemia* sp. were 43 and 32% of all PLs, respectively (Juan *et al.,* 1992). The result indicated that shrimp *A. vulgaris* showed similar ratio of PLs to those of other shrimp species. It contained 5.67 and 4.80 g/100 g of PC and PE, representing 42.63 and 36.09% of all PLs, respectively. In general, lipid classes and compositions vary among individual species and maturation stage (Sahena *et al.,* 2009; Buscailhon, *et al.,* 1994).

During *Kapi* production, TAG and ST contents gradually decreased, while both DG and FFA contents increased ($p < 0.05$). After 30 days of fermentation, FFA became dominant (49.29 g/100 g). Nevertheless, there were no differences in TAG and ST contents in F2 and F3 samples ($p > 0.05$). The results suggested that

hydrolysis or decomposition of TAG and ST took place during *Kapi* processing. Those changes were caused by both endogenous and microbial lipases. Lipase and phospholipase in shrimp play an important role in the hydrolysis of lipids (Buscailhon *et al.,* 1994). Those free fatty acids released might be susceptible to lipid oxidation during processing. Kim and Min (2008) reported that the unsaturated FFA are the most susceptible components to lipid oxidation, due to the presence of double bonds (*p*-electrons), which are less packed in lipid crystals than TAG. With increasing FFA during the process of *Kapi,* lipid oxidation could proceed more rapidly. Nevertheless, PC, PE, and OPL contents were not altered throughout the entire processes ($p > 0.05$). The results were in agreement with several previous studies, demonstrating that the degree of lipolysis of neutral lipids was higher than that of polar lipids (Bustos *et al.,* 2003; Kim and Min, 2008). During processing of French dry-cured ham for 273 days, the rate of decrease in glyceride contents of lipids was higher, compared with that of phospholipid. Coincidentally, FFA content was rapidly increased throughout the ripening period (Bustos *et al.,* 2003). Molly *et al.* (1997) also reported that during ripening of dry-fermented sausage, the degree of lipolysis in TAG fraction was higher than that of polar lipid fractions. Moreover, no changes in SE content were observed throughout the process of *Kapi* making ($p > 0.05$). The results indicated that lipids underwent hydrolysis along all processes used for *Kapi* production. Those changes more likely contributed to the final characteristics of *Kapi,* especially odor, flavor, and nutritive value.

11.5.2.2 Fatty acid compositions

Changes in FA compositions in crude lipids extracted from shrimp at all steps of *Kapi* production are presented in Table 32. In fresh shrimp, SFA was dominant, accounting for 41.82% of TFA, followed by PUFA and MUFA, which were 30.41 and 25.65%, respectively. Fresh shrimp contained 7 dominant fatty acids including 2 SFA (palmitic acid (C16:0) and stearic acid (C18:0)), 2 MUFA (palmitoleic acid (C16:1 (n-7)) and oleic acid (C18:1(n-9))) as well as 3 PUFA (arachidonic acid (AA) (C20:4(n-6)), eicosapentaenoic acid (EPA) (C20:5(n-3)) and docosahexaenoic acid (DHA) (C22:6(n-3))). Each FA constituted at levels higher than

Table 31. Lipid constituents of shrimp *A. vulgaris* during *Kapi* production

Samples[*]	SE[**]	TAG	ST	DG	FFA	PE	OPL	PC
R	5.20±1.05^{a}	28.03±0.99^{a}	24.03±0.81^{a}	7.97±1.56^{c}	11.96±1.34^{e}	4.80±0.95^{a}	2.83±1.43^{a}	5.67±1.45^{a}
S	4.60±1.04^{a}	17.87±0.91^{b}	21.73±1.92^{b}	8.97±1.27^{c}	26.87±3.18^{d}	4.20±1.91^{a}	3.10±1.39^{a}	4.57±1.56^{a}
D	4.20±1.65^{a}	8.87±0.91^{c}	17.70±1.15^{c}	13.47±1.88^{b}	34.43±1.24^{c}	3.70±1.13^{a}	2.30±1.25^{a}	6.57±1.86^{a}
F1	4.57±0.32^{a}	5.98±0.43^{d}	11.97±2.40^{d}	19.47±2.80^{a}	35.03±5.94^{c}	3.93±1.94^{a}	1.73±0.74^{a}	5.93±0.55^{a}
F2	5.30±1.11^{a}	2.50±0.30^{e}	9.63±1.25^{de}	18.80±1.47^{a}	41.33±1.56^{b}	3.67±1.40^{a}	3.37±1.31^{a}	4.63±2.25^{a}
F3	4.60±1.64^{a}	2.53±0.60^{e}	9.97±2.51^{e}	16.70±2.65^{ab}	49.29±2.07^{a}	3.53±1.07^{a}	1.90±0.80^{a}	5.33±1.95^{a}

[*]R: raw material; S: after salting; D: after drying; F1, F2 and F3: after fermentation for 10, 20 and 30 days.
[**]SE, TAG, ST, DG, FFA, PE, PC and OPL means steryl esters, triacylglycerols, sterols, diacylglycerols, free fatty acids, phosphatidylethanolamine, phosphatidylcholine, and other phospholipids, respectively.
Results are expressed as g/100 g total lipids.
Mean ± SD (n=3). Different lowercase superscripts in the same column indicate the significant difference ($p < 0.05$).

5% of total fatty acids. Fatty acid profile of shrimp *A. vulgaris* was similar to those of other shrimp including *Euphausia superba* (Kolakowska *et al.,* 1994), *Euphausia crystallorophias* (Ju and Harvey, 2004), *Macrobrachium rosenbergii* (Bragagnolo and Rodriguez-Amaya, 2001) as well as *Penaeus brasiliensis* (Bragagnolo and Rodriguez-Amaya, 2001). After salting and drying process, both SFA and MUFA contents remained constant ($p > 0.05$), while PUFA content was slightly increased ($p < 0.05$). With increasing fermentation time, PUFA content was gradually increased, whereas SFA and MUFA contents were decreased. It was postulated that some SFA and MUFA were plausibly consumed by microorganisms during fermentation. Additionally, PUFA were possibly produced by some salt tolerant microorganisms during prolonged fermentation. However, Peralta *et al.* (2008) found that the amount of PUFA of Philippine salt-fermented shrimp paste, produced from *Acetes* spp., was not significantly changed during fermentation of 360 days. Montano *et al.* (2001) also reported that fresh shrimp *Acetes* spp. had PUFA content of 31.9 g/100 g lipids. When this shrimp was used for production of *Bagoong-alamang*, a traditional shrimp paste condiments of the Philippines, the amount of PUFA was slightly increased and reached 34.7 g/100 g lipids after fermentation for 6 months. Based on our previous study, antioxidative activities of *Kapi* were increased when fermentation time increased (data not shown). Antioxidative compounds including peptides or Maillard reaction products (MRPs) probably retarded the oxidation of PUFAs during the prolonged fermentation. Thus, PUFAs underwent decomposition during processing to a lower degree. After 30 days of fermentation, FA compositions found in *Kapi* were similar to those of fresh shrimp. Moreover, the profiles were generally similar to those of Philippine fermented shrimp paste, which contained C16:0, C16:1(n-7), C18:0, C18:1(n-9), C20:4(n-6), C20:5(n-3), and C22:6(n-3) as the major fatty acids (Peralta *et al.*, 2008; Montano *et al.,* 2001). Cai *et al.* (2017) reported that Chinese low salt-fermented shrimp paste produced from wild marine shrimp (*Acetes chinensis*) had high amount of PUFAs, both EPA and DHA. Therefore, *Kapi* produced from *A. vulgaris* was rich in C20:5(n-3) (EPA) and C22:6(n-3) (DHA) and could be a good source of n-3 fatty acids.

Table 32. Changes in fatty acid composition of crude lipids of shrimp during *Kapi* production

FA composition**	R*	S	D	F1	F2	F3
Total SFA	**41.82±2.08A[a]**	**41.01±2.55A[a]**	**40.43±1.99A[ab]**	**39.52±2.03A[b]**	**39.22±2.96A[b]**	**38.39±2.13A[c]**
C8:0	ND	ND	ND	ND	0.02±0.00[b]	0.06±0.01[a]
C10:0	ND	ND	ND	0.04±0.00[b]	0.04±0.01[b]	0.06±0.02[a]
C12:0	0.22±0.02[e]	0.44±0.04[d]	0.51±0.04[c]	1.55±0.04[b]	1.88±0.03[b]	2.07±0.02[a]
C14:0	3.21±0.09[b]	3.08±0.05[c]	3.55±0.15[a]	3.06±0.09[c]	2.98±0.19[c]	2.56±0.05[d]
C16:0	27.95±2.01[a]	28.01±1.96[a]	28.03±1.56[a]	26.79±2.04[b]	26.71±2.95[b]	26.04±2.00[b]
C18:0	9.55±1.01[a]	9.01±1.55[b]	7.59±1.09[c]	7.22±0.19[cd]	7.02±0.99[d]	7.13±1.54[cd]
C20:0	0.31±0.02[ab]	0.22±0.01[b]	0.51±0.04[a]	0.56±0.02[a]	0.55±0.04[a]	0.46±.009[a]
C22:0	0.56±0.02[a]	0.25±0.01[b]	0.23±0.01[b]	0.29±0.02[b]	0.02±0.00[c]	0.01±0.00[c]
C24:0	0.02±0.00[a]	ND	0.01±0.00[a]	0.01±0.00[a]	ND	ND
Total MUFA	**25.65±1.96C[ab]**	**25.84±2.02C[ab]**	**26.16±1.55C[a]**	**25.00±1.09C[b]**	**24.72±1.02C[bc]**	**24.05±1.21C[c]**
C16:1(n-7)	11.11±0.99[b]	11.03±1.98[b]	11.25±1.44[ab]	11.59±1.00[a]	11.66±0.96[a]	11.42±1.00[a]
C18:1(n-9)	10.77±0.56[ab]	11.00±1.02[a]	10.91±1.02[a]	10.46±0.95[b]	10.09±0.55[c]	10.11±0.65[c]
C18:1(n-7)	1.44±0.04[a]	0.99±0.55[b]	1.25±0.29[ab]	0.95±0.29[b]	0.78±0.02[c]	0.65±0.09[c]
C20:1(n-9)	1.45±0.15[a]	1.48±0.04[a]	1.49±0.09[a]	1.20±0.02[b]	1.23±0.09[b]	1.02±0.09[c]
C22:1(n-11)	ND	0.20±0.01[a]	0.08±0.01[b]	0.06±0.01[b]	0.04±0.01[b]	0.05±0.01[b]
C22:1(n-9)	0.42±0.06[ab]	0.55±0.01[a]	0.58±0.01[a]	0.35±0.09[b]	0.44±0.02[ab]	0.39±0.02[b]
C24:1(n-9)	0.02±0.00[a]	0.02±0.00[a]	0.01±0.00[a]	0.02±0.00[a]	0.02±0.00[a]	0.01±0.00[a]
Total PUFA	**30.41±1.14B[d]**	**30.77±1.59B[d]**	**32.12±2.99B[c]**	**34.48±1.54B[b]**	**34.64±1.04B[ab]**	**35.54±0.95B[a]**
n-6 series	**11.1±0.65[d]**	**12.06±1.02[c]**	**12.03±2.01[c]**	**13.43±1.22[b]**	**13.58±0.77[b]**	**14.22±1.01[a]**
C18:2(n-6)	1.99±0.07[e]	2.66±0.66[d]	3.00±0.16[c]	3.48±0.55[b]	4.01±0.45[a]	4.25±0.22[a]
C18:3(n-6)	0.54±0.01[d]	1.01±0.16[c]	0.54±0.09[d]	1.52±0.02[a]	1.24±0.09[b]	1.25±0.01[b]
C20:2(n-6)	0.30±0.01[b]	0.24±0.01[bc]	0.28±0.00[b]	0.19±0.02[c]	0.20±0.01[c]	0.55±0.01[a]
C20:4(n-6) (AA)	8.06±0.09[a]	7.82±0.09[b]	8.02±0.22[a]	8.09±1.01[a]	8.02±0.95[a]	8.03±0.83[a]
C22:4(n-6)	0.21±0.01[b]	0.33±0.00[a]	0.19±0.01[bc]	0.15±0.03[c]	0.11±0.01[d]	0.14±0.02[c]
n-3 series	**19.31±0.11[b]**	**18.71±0.77[c]**	**20.09±1.01[ab]**	**21.05±2.23[a]**	**21.06±1.02[a]**	**21.32±0.55[a]**
C18:3(n-3)	0.05±0.00[d]	1.04±0.41[c]	2.02±0.23[a]	2.05±0.06[a]	1.96±0.03[b]	1.78±0.02[b]
C20:4(n-3)	ND	ND	0.02±0.00[a]	0.02±0.00[a]	0.01±0.00[a]	0.02±0.00[a]
C20:5(n-3) (EPA)	11.15±0.15[c]	12.22±0.96[ab]	12.01±1.55[b]	12.69±2.03[a]	12.55±0.99[a]	12.49±0.45[a]
C22:5(n-3)	0.23±0.00[b]	0.04±0.00[b]	0.02±0.00[b]	0.01±0.00[b]	0.02±0.00[b]	0.01±0.00[b]
C22:6(n-3) (DHA)	7.88±0.97[a]	5.41±0.02[e]	6.02±1.01[d]	6.28±0.99[cd]	6.52±0.56[c]	7.02±0.23[b]
Total fatty acids	**97.88±2.66[b]**	**97.62±2.54[b]**	**98.71±3.01[a]**	**99.00±3.02[a]**	**98.58±2.59[ab]**	**97.98±2.05[b]**

*R: raw material; S: after salting; D: after drying; F1, F2 and F3: after fermentation for 10, 20 and 30 days. Results are expressed as wt% of total fatty acids (%).

Mean ± SD (n=3). Different uppercase in the same column indicate the significant difference between SFA, MUFA and PUFA content ($p < 0.05$). Different lowercase superscripts in the same row indicate the significant difference ($p < 0.05$).

Fatty acid composition of the four major fractions (TAG, FFA, PE, and PC) in fresh shrimp and *Kapi* after 30 days of fermentation were also investigated as shown in Tables 33 and 34. For TAG fraction, SFAs were the major FAs, accounting for 51.37% of total FA, followed by MUFA (33.18%) and PUFA (13.04%), respectively. Generally, marine animals accumulate triacylglycerol or wax esters as the reserved lipids. These compounds are predominated with SFA (no double bonds) or MUFA (a single double bond), while PUFA are the minor components (Sae-leaw *et al.,* 2013). Among fatty acids of TAG fraction, C16:0 (35.55%), C18:1(n-9) (22.33%), and C18:0 (9.77%) were the major fatty acids. Some differences in fatty acid compositions of TAG fraction between fresh shrimp and resulting *Kapi* were observed. After *Kapi* processing, the amount of SFA was decreased, while both MUFA and PUFA were increased ($p < 0.05$).

For FFA fraction, different fatty acid profiles were noticeable between fresh shrimp and *Kapi.* FFA was found at the low level in fresh shrimp but became the major lipid in *Kapi* (Table 31). MUFA was the most abundant fatty acid in FFA fraction of fresh shrimp (49.65%), followed by SFA (36.29%) and PUFA (11.73%), respectively. C18:1(n-9) (25.05%) was the most abundant fatty acid found in FFA fraction of *Kapi,* followed by C16:0 (23.95%) and C16:1 (n-7) (20.22%), respectively. Similar trend to TAG fraction was observed, in which MUFA content was increased, while SFA content was decreased in *Kapi* ($p < 0.05$). However, there was no difference in PUFA content between fresh shrimp and *Kapi* ($p > 0.05$).

Fatty acid compositions of PE and PC fractions from fresh shrimp and *Kapi* were similar as shown in Table 34. Both PE and PC fractions of fresh shrimp contained PUFA as the major fatty acids, accounting for 41.31 and 45.96%, respectively. Moderate levels of SFA and MUFA were observed for both fractions (around 20-30%). It is well known that high levels of PUFA, especially n-3 PUFA, are generally found in the tissue of marine species. PLs work as cell membrane lipids and are generally rich in n-3 PUFA for membrane fluidity (Osako *et al.,* 2006).

Table 33. Fatty acid composition of TAG and FFA fractions of fresh shrimp and *Kapi* (final product)

FA composition	TAG		FFA	
	Fresh shrimp	*Kapi*	Fresh shrimp	*Kapi*
Total SFA	**51.37±3.09A^{a}**	**45.88±2.99A^{b}**	**36.29±3.04B^{A}**	**35.26±2.62A^{B}**
C8:0	ND	1.05±0.02	ND	0.5±0.02
C10:0	ND	0.07±0.01	ND	0.01±0.00
C12:0	0.51±0.04^{b}	0.95±0.09a	1.01±0.67^{A}	1.55±0.09^{A}
C14:0	3.33±0.15^{a}	2.51±0.15b	2.95±0.91^{A}	1.42±0.03^{B}
C16:0	35.55±2.99^{a}	30.05±2.96b	23.95±2.32^{B}	26.55±2.51^{A}
C18:0	9.77±2.03^{a}	10.13±2.41a	5.55±2.01^{A}	4.49±1.12^{B}
C20:0	1.19±0.92^{a}	0.57±0.19b	0.77±0.03^{A}	0.25±0.03^{B}
C22:0	0.99±0.41^{a}	0.55±0.11b	2.05±.091^{A}	0.49±0.09^{B}
C24:0	0.03±0.01	ND	0.01±0.00	ND
Total MUFA	**33.18±2.03B^{b}**	**38.42±2.28B^{a}**	**49.65±3.04A^{B}**	**52.59±3.06B^{A}**
C16:1(n-7)	5.06±1.01^{b}	8.22±1.49^{a}	20.22±2.51^{A}	19.95±2.41^{A}
C18:1(n-9)	22.33±2.66^{b}	25.65±2.19^{a}	25.05±2.69^{A}	25.63±2.52^{A}
C18:1(n-7)	1.06±0.56^{a}	0.55±0.03^{b}	2.05±1.04^{A}	1.05±0.02^{B}
C20:1(n-9)	2.55±0.24^{a}	1.05±0.02^{b}	0.33±0.02^{B}	3.04±0.08^{A}
C22:1(n-11)	ND	0.01±0.00	ND	ND
C22:1(n-9)	1.05±0.04^{a}	0.56±0.02^{b}	0.50±0.00^{A}	0.44±0.08^{A}
C24:1(n-9)	0.04±0.01^{b}	0.91±0.09^{a}	0.50±0.02^{B}	1.02±0.02^{A}
Total PUFA	**13.04±0.92C^{b}**	**14.39±0.24C^{a}**	**11.73±0.55C^{A}**	**11.32±0.98C^{A}**
n-6 series	**3.92±0.56^{b}**	**4.89±0.02^{a}**	**5.57±0.16^{A}**	**2.78±0.22^{B}**
C18:2(n-6)	1.05±0.08^{a}	1.02±0.02^{a}	2.22±0.22^{A}	0.22±0.01^{B}
C18:3(n-6)	0.25±0.02^{a}	0.02±0.00^{b}	0.50±0.22^{A}	0.35±0.02^{B}
C20:2(n-6)	0.02±0.00^{a}	0.01±0.00^{a}	0.05±0.02^{A}	0.01±0.00^{A}
C20:4(n-6) (AA)	2.05±0.02^{b}	3.55±0.05^{a}	2.55±0.14^{A}	2.05±0.12^{B}
C22:4(n-6)	0.55±0.44^{a}	0.29±0.02^{b}	0.25±0.11^{A}	0.15±0.02^{B}
n-3 series	**9.12±0.99^{a}**	**9.50±0.25^{a}**	**6.16±1.52^{B}**	**8.54±1.03^{A}**
C18:3(n-3)	0.06±0.02^{b}	0.44±0.02^{a}	1.01±0.55^{A}	0.22±0.02^{B}
C20:4(n-3)	ND	0.01±0.00	ND	0.02±0.00
C20:5(n-3) (EPA)	2.25±0.04^{b}	3.55±0.19^{a}	0.43±0.22^{B}	1.65±0.03^{A}
C22:5(n-3)	0.16±0.02^{a}	0.01±0.00^{b}	0.22±0.12^{A}	0.01±0.00^{B}
C22:6(n-3) (DHA)	6.65±0.45^{a}	5.49±0.11^{b}	4.50±0.91^{B}	6.64±0.10^{A}
Total fatty acids	**97.59±3.01^{b}**	**98.69±2.48^{a}**	**97.67±3.33^{B}**	**99.17±3.02^{A}**

Results are expressed as wt% of total fatty acids (%).
Mean ± SD (n=3). Different uppercase in the same column indicate the significant difference between SFA, MUFA and PUFA content ($p < 0.05$). Different lowercase and uppercase superscripts in the same row within the same lipid class indicate the significant difference ($p < 0.05$).

Table 34. Fatty acid composition of PE and PC fractions of fresh shrimp and *Kapi* (final product)

FA composition	PE		PC	
	Fresh shrimp	***Kapi***	**Fresh shrimp**	***Kapi***
Total SFA	**28.25±2.95B^{a}**	**26.16±2.50C^{b}**	**29.17±1.99B^{A}**	**29.81±2.35B^{A}**
C8:0	ND	ND	ND	ND
C10:0	ND	ND	ND	ND
C12:0	0.63±0.06^{a}	0.55±0.02^{a}	2.56±0.43^{A}	2.05±0.14^{A}
C14:0	0.55±0.02^{b}	0.92±0.05^{a}	2.05±0.45^{A}	2.03±0.22^{A}
C16:0	22.02±2.59^{a}	19.66±2.44^{b}	18.25±2.01^{B}	20.33±2.31^{A}
C18:0	1.55±0.91^{b}	2.06±0.11^{a}	0.59±0.02^{A}	0.45±0.09^{A}
C20:0	1.05±0.22^{a}	0.92±0.19^{a}	2.22±0.06^{A}	2.01±0.09^{A}
C22:0	2.44±0.94^{a}	2.05±0.05^{b}	3.50±0.14^{A}	2.94±0.11^{B}
C24:0	0.01±0.00	ND	ND	ND
Total MUFA	**28.44±2.02B^{b}**	**29.03±3.02B^{a}**	**22.64±3.05C^{A}**	**20.68±2.99C^{B}**
C16:1(n-7)	13.95±2.01^{a}	13.33±2.44^{a}	12.23±2.29^{A}	10.22±2.06^{B}
C18:1(n-9)	10.35±1.16^{b}	12.65±2.68^{a}	8.83±2.95^{B}	10.13±2.93^{A}
C18:1(n-7)	2.65±0.02^{a}	2.02±0.12^{b}	0.55±0.05^{A}	0.24±0.02^{B}
C20:1(n-9)	0.55±0.02^{b}	0.95±0.09^{a}	0.09±0.01^{A}	0.05±0.01^{A}
C22:1(n-11)	ND	ND	ND	ND
C22:1(n-9)	0.22±0.02^{a}	0.02±0.00^{b}	0.22±0.02^{A}	0.01±0.00^{B}
C24:1(n-9)	0.25±0.02^{a}	0.02±0.00^{b}	0.25±0.06^{A}	0.01±0.00^{B}
Total PUFA	**41.31±2.52A^{b}**	**42.58±1.59A^{a}**	**45.96±2.56A^{B}**	**47.16±3.44A^{A}**
n-6 series	17.68±2.33^{b}	19.68±1.22^{a}	24.31±2.41^{B}	27.63±2.99^{A}
C18:2(n-6)	3.65±1.92^{a}	3.77±0.03^{a}	3.55±1.13^{B}	4.55±1.15^{A}
C18:3(n-6)	0.44±0.03^{a}	0.35±0.02^{a}	0.91±0.09^{A}	1.01±0.02^{A}
C20:2(n-6)	0.32±0.02^{a}	0.45±0.03^{a}	0.01±0.00^{A}	0.03±0.01^{A}
C20:4(n-6) (AA)	13.25±0.99^{b}	15.06±1.18^{a}	20.22±2.91^{B}	22.02±3.01^{A}
C22:4(n-6)	0.02±0.00^{b}	0.05±0.01^{a}	0.02±0.00^{A}	0.02±0.01^{A}
n-3 series	23.63±2.09^{a}	22.90±2.06^{b}	21.65±2.25^{A}	19.53±2.55^{B}
C18:3(n-3)	10.23±2.00^{a}	9.44±2.04^{b}	15.15±2.09^{A}	13.44±2.41^{B}
C20:4(n-3)	ND	ND	ND	ND
C20:5(n-3) (EPA)	4.63±1.14^{b}	5.55±0.55^{a}	2.05±0.41^{A}	1.93±0.23^{B}
C22:5(n-3)	0.22±0.02^{a}	0.20±0.01^{a}	0.01±0.00^{A}	0.01±0.00^{A}
C22:6(n-3) (DHA)	8.55±1.25^{a}	7.71±2.03^{b}	4.44±0.02^{A}	4.15±0.02^{A}
Total fatty acids	**98.00±3.02^{a}**	**97.77±2.96^{a}**	**97.77±3.56^{A}**	**97.65±3.22^{A}**

Results are expressed as wt% of total fatty acids (%).
Mean ± SD (n=3). Different uppercase in the same column indicate the significant difference between SFA, MUFA and PUFA content ($p < 0.05$). Different lowercase and uppercase superscripts in the same row within the same lipid class indicate the significant difference ($p < 0.05$).

Among all PUFA in both fractions, C20:4(n-6) (AA), C18:3(n-3), C20:5(n-3) (EPA), and C22:6(n-3) (DHA) were dominant. Although the major fatty acids of PE fraction were similar to those of PC fraction, slightly higher n-3 PUFA levels (both EPA and DHA) were found in PE fraction, compared to those of PC fraction. However, the higher n-6 PUFA levels, especially AA (C20:4 (n-6)), were obtained in PC fraction, compared to PE fraction. In general, total amount of these PUFA in both fractions were slightly increased as fermentation proceeded ($p < 0.05$). This result confirmed that processing and fermentation did not negatively affect fatty acid composition, in which PUFA were still retained. Thus *Kapi* was a rich source of PUFA, both n-3 and n-6 series, offering the benefits to human health.

11.5.3 Change in lipid oxidation products of shrimp during *Kapi* production

Peroxide and TBARS values were used as indices to assess the level of lipid oxidation in shrimp during *Kapi* production (Figure 31). PV of fresh shrimp was about 0.14 mg hydroperoxide/kg dry sample, indicating that oxidation of lipid occurred after capture or during transportation prior to processing. A continuous increase in PV was observed when shrimp were subjected to salting and drying ($p < 0.05$). Pounding shrimp during salting and drying processes plausibly increased the surface area of the shrimp meat. As a result, lipids were liberated and exposed to oxygen, in which oxidation could be accelerated (Visessanguan *et al.,* 2006). PV was drastically increased during the first 10 days of fermentation and remained unchanged after 20 days of fermentation. Subsequently, the decrease in PV was noticeable as fermentation proceeded up to day 30. The increase in PV during *Kapi* production was more likely due to the formation of hydroperoxide. Oxidation governed by several intrinsic factors, e.g., pro-oxidants, fatty acid composition, etc. (Visessanguan *et al.,* 2006). The decrease in PV observed during extended fermentation time was due to the decomposition of hydroperoxide to the secondary oxidation products (Sea-leaw *et al.,* 2013). The result indicated that lipid oxidation occurred during *Kapi* production. It was noted that oxidation occurred to a small extent as indicated by the high level of PUFA in *Kapi* (Table 3). These lipid oxidation products could affect odor and flavor characteristics of *Kapi.*

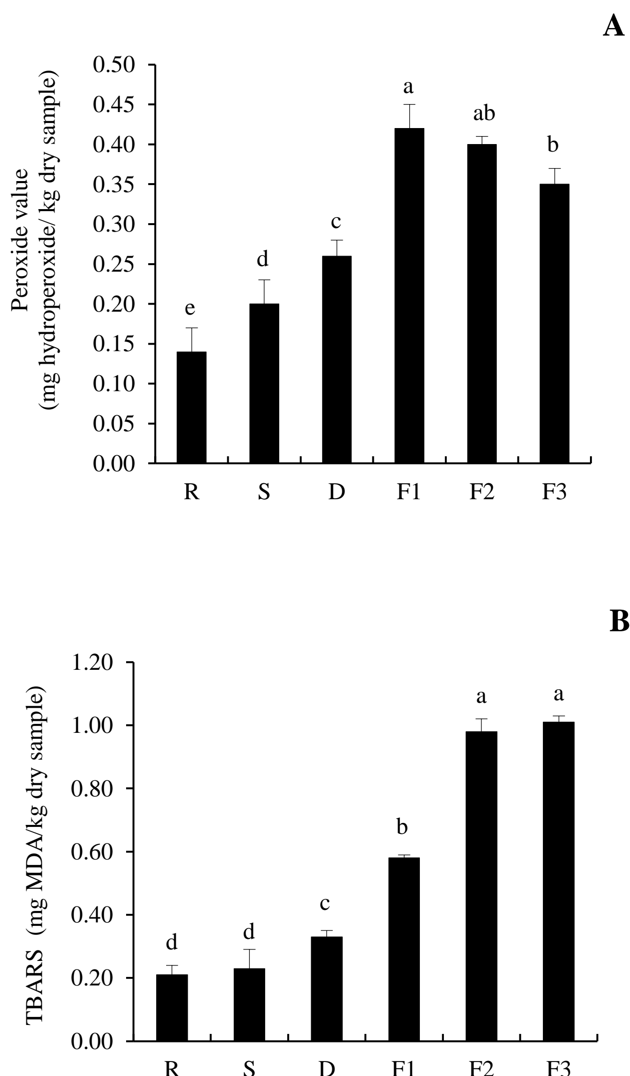

Figure 31. Peroxide value (A) and TBARS value (B) of shrimp during *Kapi* production. R: raw material; S: after salting; D: after drying; F1, F2 and F3: after fermentation for 10, 20 and 30 days.

Bars represent the standard deviation from triplicate determinations. Different letters on the bars denote the significant differences ($p < 0.05$).

The initial value of TBARS was 0.21 mg MDA/kg dry sample. Those secondary oxidation products could be formed during post-mortem handling or storage of shrimp used as raw material for *Kapi* making. No change in TBARS was observed after salting ($p > 0.05$). TBARS value was increased after sun-drying and it was continuously increased within the first 20 days of fermentation ($p < 0.05$). No difference in TBARS was found when fermentation was performed for 20 and 30 days ($p > 0.05$).

TBARS value is the good index of relatively polar secondary reaction products, especially aldehydes (Sea-leaw *et al.,* 2013).

Overall, during *Kapi* production, lipid oxidation proceeded in shrimp as evidenced by the increases in PV and TBARS values. During fermentation, autolysis or endogenous enzymatic activity might cause the disruption of the organelles associated with the release of pro-oxidants as well as reactants (Takeungwongtrakul *et al.,* 2012). This was associated with lipid oxidation taking place in *Kapi.* Lipid oxidation is one of the deteriorative reactions causing the unacceptability of fish and shrimp product (Visessanguan *et al.,* 2006). However, no objectionable odor or tastes were obtained in all samples throughout *Kapi* production/fermentation (data not shown). On the other hand, those lipid oxidation products might contribute to the typical flavor or odor of *Kapi.*

11.6 Conclusion

Shrimp *A. vulgaris*, a by-catch from commercial fishing, has become alternative source for *Kapi* production. Lipid fractions of fresh shrimp consisted of TAG, ST, PE, and PC as the dominant lipids. SFA and MUFA were the dominant fatty acids found in TAG, while PE and PC were rich in PUFA, especially C20:5(n-3) (EPA). An increase in lipolysis, indicated by the increased FFA with coincidentally decreased TAG, was observed throughout *Kapi* production. An increase in lipid oxidation was also noticeable as evidenced by the increases in PV and TBARS. These changes could be related with the final characteristics of the product, especially odor and flavor.

11.7 References

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CHAPTER 12

BACILLUS SPP. K-C3 ISOLATED FROM *KAPI*, SALTED SHRIMP PASTE OF THAILAND: ITS EXTRACELLULAR ENZYMES AND THE USE AS STARTER CULTURE IN *KAPI*

12.1 Abstract

The growth and production of extracellular enzymes including protease, lipase as well as chitinase of *Bacillus* spp. K-C3, isolated from commercial *Kapi*, were determined. Growth increased exponentially up to 24 h, followed by stationary phase. Protease production reached the maximum level (159.23 U/ml) during the late stationary phase (48 h), while drastic increases in both lipase and chitinase production were obtained during the early stationary phase (24 h) ($p < 0.05$), which were 105.48 and 75.03 U/ml, respectively. Protease secretion was corresponded with the growth, which was optimal at 35°C and pH 8.0. Nevertheless, the maximal lipase and chitinase secretion was found at pH 7.0, 25°C and pH 6.0, 30-35°C, respectively. Moreover, the highest growth and enzyme activities were obtained in the presence of 10% (w/v) salt. Changes in enzyme activities in *Kapi,* inoculated without and with *Bacillus* spp. K-C3 at different level (10^2, 10^4 and 10^6 CFU/g dry weight sample), during *Kapi* production, were monitored. For *Kapi* without inoculation (control), *Bacillus* spp. K-C3 existed after drying process and remained throughout fermentation of 30 days as examined by polymerase chain reaction denaturing gradient gel electrophoresis (PCR-DGGE) technique. For all inoculated samples, *Bacillus* spp. K-C3 bands were also detected in all steps of *Kapi* processing, indicating that this strain could survive during the entire process. All inoculated samples generally exhibited the higher proteolytic, lipolytic and chitinolytic activities, compared to the control. Therefore, *Bacillus* spp. K-C3 could be used as starter culture, which increased enzyme activities during *Kapi* production and enhanced the fermentation of *Kapi.*

12.2 Introduction

Kapi, a traditional salted shrimp paste of Thailand, is one of the most popular condiment used in many Thai dishes. *Kapi* is made from small shrimp/krill mixed with solar salt, followed by sun-drying to reduce the moisture and grinding into paste. The paste is allowed to ferment in the earthen jar for at least 1 month at room temperature (28-30°C), in which the typical aroma is fully developed (Pongsetkul *et al.,* 2017a). Both proteolysis and lipolysis are the important biochemical changes occurring throughout *Kapi* processing, especially during fermentation (Pongsetkul *et al.,* 2017a, c). During fermentation, hydrolysis of proteins and lipids are induced by endogenous enzymes in shrimp/krill as well as microbial enzymes surviving under high salt condition (Gildberg and Stenberg, 2001). Proteolysis influences both texture and flavor development since it can induce the formation of several low molecular weight compounds including peptides, amino acids, aldehydes, organic acids and amines (Visessanguan *et al.,* 2006). Lipolysis also plays a major role in quality of *Kapi,* associated with the formation of free fatty acids liberated, which further undergo oxidation. Those oxidation products, e.g., aldehydes and ketones, are responsible for the development of typical flavor or aroma (Itou *et al.,* 2006). Therefore, proteolysis and lipolysis directly affect the final *Kapi* characteristics.

The use of starter cultures is widespread in various kinds of fermented foods since they have a significant impact on food attributes such as taste, texture, odor and nutritive value (Visessanguan *et al.,* 2006). Among starter cultures, *Bacillus* sp. seem to have the potential for many fermented products due to their ability to hydrolyze protein and lipid (Chantawannakun *et al.,* 2002; Sanni *et al.,* 2002; Wittanalai *et al.,* 2010). *Bacillus* spp., including *B. amyloliquefaciens* and *B. subtilis,* have been reported as safe hosts for the production of harmless and safe food products (Boer and Diderichsen, 1991). They are considered as the Generally Recognized as Safe (GRAS) (Ferreira *et al.,* 2005).

Bacillus spp., isolated from *Kapi*, also exhibited the strong proteolytic as well as fibrinolytic activities. These strains worked well at the beginning of the fermentation but produced a putrid odor in the later stages of fermentation (Chotwanawirach, 1980). Surono and Hosono (1994) reported that the microflora of

terasi, the Indonesian shrimp paste, were *Bacillus*, *Pseudomonas*, *Micrococcus, Kurthia* and *Sporolactobacillus*. Among these strains, more than 50% of all isolates belonged to aerobic spore-forming *bacilli,* and the dominant strains were identified as *Bacillus brevis*, *B. sphaericus*, *B. subtilis* and *B. circulans*. Enzymes from these strains were mainly responsible for deamination and decarboxylation of amino acids as well as for the formation of lower MW fatty acids and amides, thus producing characteristic flavor of terasi. Moreover, Aryanta (2000) documented that halophiles, belonging to *Bacillus* spp., isolated from *terasi,* could possibly hydrolyze short-chain fatty acids, responsible for the cheesy odor of the product.

Recently, some microorganisms from commercial *Kapi* were isolated. Among those strains, *Bacillus* spp. K-C3 exhibited the highest proteolytic activity. Therefore, this strain seemed to have the high potential as starter culture for *Kapi* production. However, their growth and ability to produce extracellular enzymes including protease, lipase and chitinase, during *Kapi* processing has not been studied.

12.3 Objective

To monitor extracellular enzymes including protease, lipase as well as chitinase in *Kapi* inoculated with *Bacillus* spp. K-C3. The growth of *Bacillus* spp. K-C3 and their extracellular enzyme production were also examined.

12.4 Materials and methods

12.4.1 Bacterial strain

Lyophilized culture of *Bacillus* spp. K-C3, isolated from commercial *Kapi,* was obtained from the Food microbiology laboratory of Prince of Songkla University (Thailand). The strain was subcultured twice in 0.5% nutrient broth (NB) at 37.5°C for 24 h under shaking condition (180 rpm). Broth culture was diluted to obtain 10^7 CFU/ml as estimated by the absorbance at 600 nm and used as an inoculum.

12.4.2 Growth and production of extracellular enzymes from *Bacillus* spp. K-C3

12.4.2.1 Cultivation and collection of bacteria

Growth and production of extracellular enzymes from *Bacillus* spp. K-C3 were monitored as a function of time. *Bacillus* spp. K-C3 was cultivated by adding 1 ml of bacterial suspension (10^7 CFU/ml) into 50 ml of 0.5% NB containing 10% NaCl and 0.8% substrates (casein for protease, palm oil for lipase (0.01% gum arabic was also added as emulsifier) or colloidal chitin for chitinase). The mixture was incubated at 37.5°C under shaking condition (180 rpm). During cultivation of 72 h, samples were collected aseptically every 4 h and 12 h for measuring cell density (OD_{600}) and production of extracellular enzymes, respectively. To determine enzyme activities, the culture was centrifuged at 10,000×*g* for 5 min at 4°C using the refrigerated centrifuge (Beckman Coulter, Avanti J-E Centrifuge, Palo Alto, CA). Cell-free supernatants were collected and considered as crude extracts. They were further subjected to determination of enzyme activities.

12.4.2.2 Determination of extracellular enzyme activities

- Proteolytic activity assay

Proteolytic activity of crude extract was assayed according to the method of Klomklao *et al.* (2008). Crude extract (200 µl) was added into assay mixtures containing 2 mg of casein, 200 µl of distilled water and 625 µl of 20 mM Tris-HCl buffer (pH 8.0). The mixture was incubated at 60°C for precisely 20 min. Reaction was terminated by adding 200 µl of 50% cold-TCA (w/v). Unhydrolyzed protein was allowed to precipitate for 1 h at 4°C, followed by centrifuging at 10,000×*g* for 10 min. The oligopeptide content in the supernatant was determined by the Lowry assay (Lowry *et al.,* 1951) using tyrosine as a standard. A blank was run in the same manner, except the crude extract was added after addition of cold 50% TCA (w/v) (0-2°C). The activity (1 unit) was defined as the enzyme causing the release of 1 µmole of tyrosine equivalent/min/ml of crude extract.

- Lipolytic activity assay

Lipolytic activity was determined using *p*-nitrophenyl palmitate (*p*-NPP) as a substrate according to the method of Sae-leaw and Benjakul (2018). *p*-NPP was dissolved in methanol to obtain a concentration of 15 mM and used as a stock solution. Substrate working solution was 0.25 mM *p*-NPP in 20 mM Tris-HCl buffer (pH 8.0) containing 20 mM $CaCl_2$, 5 mM sodium cholate and 0.01% gum arabic. The substrate solution was left at 30°C for 20 min before the assay. Crude extract with an appropriate dilution (20 µl) was added into 3.0 ml of substrate working solution. The reaction mixture was incubated at 30°C for precisely 5 min. The release of *p*-nitrophenol (*p*-NP) was measured at 410 nm using a UV-1601 spectrophotometer (Shimadzu, Kyoto, Japan). The activity (1 unit) was defined as the amount of enzyme producing 1 µmole of *p*-NP equivalent/min/ml of crude extract.

- Chitinolytic activity assay

Chitinolytic activity was determined colorimetrically by detecting the amount of *N*-acetylglucosamine (GlcNAc) released from a colloidal chitin substrate (Thamthiankul *et al.,* 2001) with some modifications. The reaction mixture containing 1 ml of 1% (w/v) colloidal chitin (in 0.02 M phosphate buffer, pH 5.2) and 1 ml of diluted crude enzyme solution was incubated at 37°C for precisely 30 min. Thereafter, 3 ml of 3,5-dinitrosalicylic acid (DNS) reagent were added. The mixture was boiled for 10-15 min. After cooling, the developed color, as the indicator of releasing GlcNAc, was measured at 530 nm. Standard curve was prepared using 0-10 ppm of GlcNAc (Sigma, USA). The chitinolytic activity (1 unit) was defined as the amount of enzyme producing 1 µmole of GlcNAc/min/ml of crude extract.

12.4.3 Optimal condition for bacterial growth and extracellular enzyme production

For all activities, 1 ml of inoculum (10^7 CFU/ml) was transferred into 50 ml of 0.5% NB.

12.4.3.1 Effect of pH

The effect of pH on extracellular enzyme production was determined by culturing the strain in 0.5% NB containing 10% NaCl at different pHs (4-11) using various buffers, including 0.1 M sodium acetate buffer for pH 4.0-5.0, 0.1 M potassium phosphate buffer for pH 6.0-8.0, 0.1 M Tris-HCl buffers for pH 8.0-9.0 and 0.1 M glycine-NaOH buffer for pH 9.0-12.0 (Nedra El-Hadj *et al.,* 2007). After incubation for 24 h at 37.5°C under shaking condition at 180 rpm, all enzyme activities and microbial growth were quantified.

12.4.3.2 Effect of temperature

The effect of temperature on growth and extracellular enzyme production was studied by culturing bacteria in 0.5% NB containing 10% NaCl. Incubation was performed at different temperatures (25-60°C) under shaking condition at 180 rpm. After 24 h, extracellular enzyme activities as well as their growth were measured.

12.4.3.3 Effect of NaCl concentration

To investigate the effect of NaCl, NaCl was added into 0.5% NB to obtain the final concentrations of 0, 5, 10, 15, 20, 25 and 30% (w/v). The growth and extracellular enzyme activities were determined after incubation of 24 h at 37.5°C under shaking condition at 180 rpm.

12.4.4 Changes in enzyme activities of shrimp inoculated with *Bacillus* spp. K-C3 during *Kapi* production

12.4.4.1 Preparation of *Kapi*

Shrimp *Acetes vulgaris* (average body length 15.6±1.4 mm, average wet weight 0.0413±0.0098 g, n=20) obtained from the local market in Ko-yo, Songkhla, Thailand, were used as raw material for making *Kapi.* Ten kg of shrimp were washed with 3% NaCl solution, drained and placed into the polypropylene basket. Solar salt was added with the shrimp: salt ratio of 5:1 (w/w), mixed well and then allowed to stand

overnight at room temperature (28-30°C). Salted shrimp were drained and ground into uniformity using a blender (National, Tokyo, Japan). Subsequently, salted shrimp were spread out on the fiberglass mats and allowed to dry with sunlight until the moisture content was in the range of 35-40% as determined by AOAC method (AOAC 2000). Thereafter, dried shrimp were inoculated with the bacterial suspension to obtain approximate cell concentrations of 10^2, 10^4 and 10^6 CFU/g dried weight sample, mixed well and then impacted into earthen jars. Jars were covered with the cheese cloth. The fermentation was proceeded at room temperature (28-30°C) for 1 month. The control was prepared without inoculum. During *Kapi* production, samples were collected at different steps of production as follows: (1) fresh shrimp, (2) after salting, (3) after drying and (4) during fermentation of 10, 20 and 30 days. Each samples were subjected to determination of microbial count as well as enzyme activities.

12.4.4.2 Monitoring of *Bacillus* spp. K-C3 during *Kapi* production

To monitor the presence of *Bacillus* spp. K-C3 during production, polymerase chain reaction denaturing gradient gel electrophoresis (PCR-DGGE) analysis was carried out using the Dcode universal mutation detection system apparatus (Cleaver Scientific, Rugby, Warwickshire, UK) as described by Sanchart *et al.* (2015). Before analysis, *Bacillus* spp. K-C3 strain and *Kapi* samples during production were subjected to DNA extraction. For *Kapi* samples, 10 g of samples were extracted using 90 ml of distilled water. The mixture were centrifuged at 14,000×g for 10 min at 4°C to pellet the cells. The pellet was washed twice with 1 ml of sterile 0.85% (w/v) NaCl and subjected to DNA extraction using GF-1 bacterial DNA extraction kits (VIVANTIS, Selangor, Malaysia). For *Bacillus* spp. K-C3 strain, DNA of 1 ml strain suspension (10^7 CFU/ml) were subjected to centrifugation and the pellet were extracted using DNeasy mericon food kit (QIAGEN, Hilden, Germany). An approximately 1500 bp fragment of the 16S rDNA was amplified using forward primer 27f (5' AGAGTTTGATCMTGGCTCAG 3') and reverse primer 1492r (5' TACGGY TACCTTGTTACGACTT 3'). Then, PCR products were amplified again with primers 341f (5′-CCTACGGGAGGCAGCAG-3′) and 518r (5′ATTACCGCGGCTGCTGG-3′) to obtain a region of approximately 200 bp of the V3 region of 16S rDNA. A GC-clamp

(CGCCCGCCGCGCGCGGCGGGCGGGGCGGGGGCACGGGGGG) was applied to the 5′ end of the forward primer to increase the sensitivity of the DGGE analysis (Sanchart *et al.,* 2015). PCR products of *Bacillus* spp. K-C3 strain and *Kapi* during production were subjected to DGGE analysis.

For DGGE analysis, 30 µl of PCR product were applied onto 8% (w/v) polyacrylamide gels (30-60% denaturing gradient). Electrophoresis was run in 1X TAE buffer at constant temperature (60°C) at 20 V for 10 min and subsequently at 120 V for 6 h. Finally, the gel was stained for 10 min with 1X SYBR Gold (Invitrogen, Carlsbad, CA, USA), and visualized and photographed under UV illumination with the Gel Documentation apparatus (UVI-TECH, Cambridge, UK).

12.4.4.3 Change in total viable count (TVC) during *Kapi* production

Samples (25 g), collected during *Kapi* production, were aseptically transferred into a stomacher bag containing 225 ml of 0.1% sterile peptone water containing 10% NaCl and pummeled for 3 min at 200 rpm using a stomacher 400 Lab Blender (Seward Ltd., Worthing, UK). Appropriate decimal dilutions of the suspension were prepared using the same diluent and 0.1 ml of each dilution was plated in triplicate on plate count agar containing 10% NaCl (Oxoid Ltd., Hampshire, England) and incubated at 35°C for 48 h (BAM 2001). TVC was expressed as log CFU/g dry weight sample.

12.4.4.4 Changes in enzyme activities during *Kapi* production

Shrimp during *Kapi* production was collected and crude extract was prepared as per the method of Klomklao *et al.* (2008). Sample (20 g) was suspended in 200 ml of 20 mM Tris-HCl buffer (pH 8.0) containing 10 mM $CaCl_2$. The mixture was homogenized for 2 min using an IKA Labortechnik homogenizer (Selangor, Malaysia) at a speed of 11,000 rpm. The homogenate was stirred continuously at 4°C for 3 h, followed by centrifugation at 8,000×*g* for 30 min using a refrigerated centrifuge. All procedures were carried out at 4°C. The supernatants considered as crude extracts were determined for proteolytic, lipolytic and chitinolytic activities.

12.4.5 Statistical analysis

All experiments were run in triplicate using three different lots of samples. The data were subjected to analysis of variance (ANOVA). Statistical analysis was done using one-way analysis of variance (ANOVA). Mean comparison was conducted using the Duncan's multiple range test (Steel *et al.,* 1980). Analysis was performed using the SPSS package (Version 10.0) (SPSS for windows, SPSS Inc., Chicago, II, USA).

12.5 Results and discussion

12.5.1 Growth and production of extracellular enzymes from *Bacillus* spp. K-C3

Growth of *Bacillus* spp. K-C3 and activities of extracellular enzymes including protease, lipase as well as chitinase as a function of time are depicted in Figure 32. Casein, palm oil and colloidal chitin were used as substrates for protease, lipase and chitinase, respectively. Overall, growth of *Bacillus* spp. K-C3 in the presence of different substrates were not different. It was noted that *Bacillus* spp. K-C3 had the exponential growth within the first 4 h and rapidly grew up to 24 h, followed by stationary phase during 24-48 h. The death phase as indicated by the sharp decrease in the intensity of OD_{600} was found after 48 h. Lopez *et al.* (2009) reported that mostly *Bacillus* spp., especially *Bacillus subtilis,* has a doubling time of 30 min during their growth and the typical four phases were normally obtained within 2-3 days. Patel *et al.* (2005) also revealed that *Bacillus* spp. had the exponential growth phase within 2 h.

Normally, *Bacillus* has been known as the excellent source of protease in many fermented food, e.g., *Bacillus simplex* in Korean salt-fermented anchovy (Ha *et al.,* 2002) or *Bacillus megaterium* in Thai fish sauce (Yossan *et al.,* 2006), etc. *Bacillus* spp. K-C3 was found to secrete proteases (Figure 1A). After 12 h, the proteolytic activity of *Bacillus* spp. K-C3 was 8.85 U/ml crude extract. The marked increase in protease activity was observed when the incubation time increased and reached the highest value at 48 h (159.23 U/ml crude extract) ($p < 0.05$). The result indicated that *Bacillus* spp. K-C3 could produce and secrete extracellular proteases at

their later stationary phase. Patel *et al.* (2005) documented that haloalkaliphilic *Bacillus* sp., Ve1, produced substantial levels of extracellular alkaline protease within the early stationary phase of their growth. However, a major portion of proteases was secreted by *B. sphaericus* in the post exponential phase of their growth (Ferrero *et al.,* 1996). The slight decrease in protease secretion from *Bacillus* spp. K-C3 was observed after 48 h of incubation, but it was still high up to 72 h. Therefore, this *Bacillus* strain had the potential to produce extracellular proteases within 2 day and protease activity was still remained up to 3 days.

Bacillus spp. K-C3 also produced extracellular lipases as indicated by lipolytic activity detected in the broth containing palm oil as a substrate as depicted in Figure 1B. Lipase produced by *Bacillus* spp. K-C3 at the incubation time of 12 h was 95.33 U/ml crude extract. The activity was slightly increased as the incubation time increased ($p < 0.05$). The highest lipolytic activities were found at the incubation time of 24 and 36 h (105.48 and 103.99 U/ml crude extract, respectively) ($p < 0.05$). Thereafter, slight decrease in activity was obtained as incubation time increased. However, lipase from *Bacillus* spp. K-C3 detected in the broth was still high up to 72 h. The result revealed that *Bacillus* spp. K-C3 could produce and secrete extracellular lipases in the post exponential phase of their growth. Lipase were still secreted during their stationary phase and death phase at the lower extent. El-moniem Abada (2008) reported that *Bacillus stearothermophilus* AB-1 secreted extracellular lipases within 4 h of cultivation, but the highest lipase secretion was found after incubation of 48 h. Lipase secretion from *Bacillus* sp. (SRBIT-05), isolated from oil mill soil, was observed within the first 2 h and quite stable throughout the cultivation up to 96 h. This strain exhibited the highest lipase production after cultivation for 36 h as reported by Ramesh *et al.* (2014).

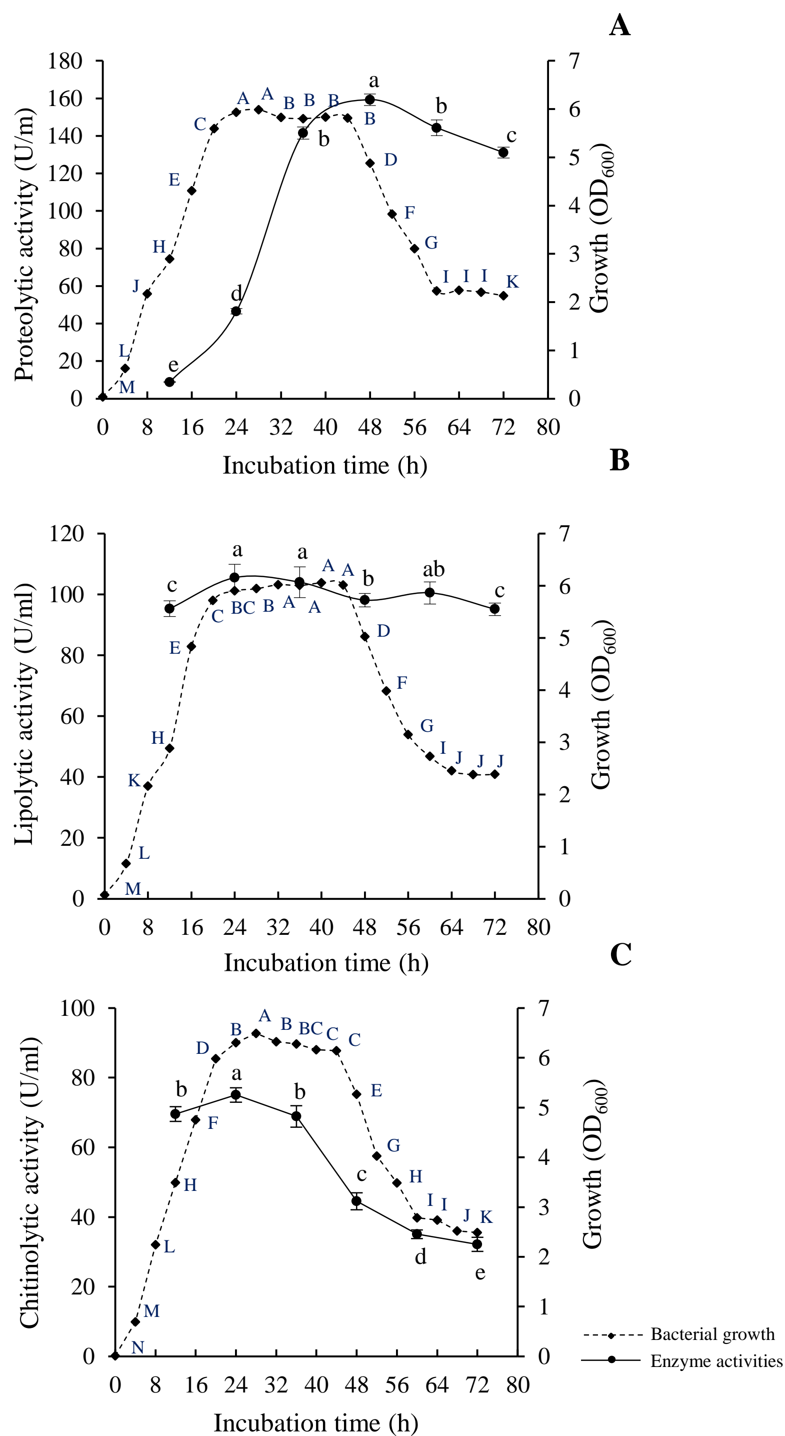

Figure 32. Growth and extracellular enzymes from *Bacillus* sp. K-C3 including proteolytic activity (A), lipolytic activity (B) and chitinolytic activity (C) as a function of time.

Bars represent the standard deviation from triplicate determination. Different uppercase and lowercase letters indicate the significant differences ($p < 0.05$).

Figure 32C shows that the *Bacillus* spp. K-C3 strain was able to secrete extracellular chitinases. The chitinolytic activity at the first 12 h of incubation was 69.55 U/ml crude extract, indicating that this strain was able to produce and secrete chitinase during their post exponential phase. The highest chitinolytic activity was found when incubated for 24 h ($p < 0.05$), which was the early stationary phase of the growth. However, the secretion of chitinase was drastically decreased as incubation time increased and the activity was only 32.16 U/ml crude extract after incubation for 72 h. The result indicated the reduction of chitinase secretion during the death phase of *Bacillus* spp. K-C3. Moreover, the secreted chitinase was not stable, as indicated by the loss in activity. *Bacillus cereus* TKU027, isolated from soils in the northern Taiwan, exhibited the chitinolytic activity over the incubation time of 4-48 h (Wang *et al.,* 2012). *Bacillus subtilis* SL-13, isolated from processed tomato, was grown for 24 h to achieve stationary phase and after an incubation of 5 h, the chitinase activity was detected at the high level. Chitinase secretion increased with increasing incubation time up to 40 h (Yan *et al.,* 2011).

Thus, *Bacillus* spp. K-C3 was able to produce protease, lipase and chitinase in the presence of corresponding substrates. The production of those enzymes depended on the incubation time.

12.5.2 Optimal condition of *Bacillus* spp. K-C3 growth and its extracellular enzyme production

12.5.2.1 Effect of pH

Figure 33A shows the growth and the production of extracellular enzymes over the pH range of 4-11. The growth of *Bacillus* spp. K-C3 was very low at the acidic pH (4-5). At very acidic pH, the solubility of various metals was high. Those metals easily cross the bacterial membrane, resulting in undesirable redox reactions. These reactions could breakdown the cell membrane and led to the cell death (Varela and Tein, 2003). *Bacillus* spp. K-C3 could grow well in the pH range of 6-9 with an optimum at 8. This was similar to other *Bacillus* strains. *Bacillus* sp. Ve1 showed the maximum growth at pH 8-9 and the growth was substantially reduced at pH below 7 (Patel *et al.,* 2005). The optimal pH for the growth of *Bacillus alveayuensis*

CAS 5, isolated from marine sediments of *Parangipettai* was 9 (Annamalai *et al.,* 2014), while *Bacillus subtilis* (strain WT 168) grew well over the pH range of 6.5-8.5 and exhibited the highest growth at pH 8 as reported by Krulwich *et al.* (1985). The growth of *Bacillus* spp. over the entire range of pH of 5-9.5, particularly alkaline pH ranges, might be due to cytoplasmic buffering capacities of this strain (Krulwich *et al.,* 1985). Microorganisms belonging to *Bacillus* species generally grow over several environmental pH, while maintaining cytoplasmic pH within a relatively narrow range that stabilizes protein and nucleic acid (Cotter and Hill, 2003). The result suggested that *Bacillus* spp. K-C3 could be classified as neutrophile, in which they grow optimally within one or two pH units of the neutral pH (Krulwich *et al.,* 1985).

When *Bacillus* spp. K-C3 was incubated at the acidic pH range, the secretion of all extracellular enzymes including protease, lipase as well as chitinase, was very low, particularly at pH 4 and 5. However, the higher level of enzyme secreted was found when the pH of the medium was increased ($p < 0.05$). The maximal proteolytic activity was attained at pH 8 (150.03 U/ml crude extract), followed by pH 7 (135.44 U/ml crude extract) and pH 9 (130.19 U/ml crude extract), respectively ($p < 0.05$). This indicated that *Bacillus* spp. K-C3 could secrete extracellular proteases at high level when the pH was slightly alkaline. For both lipase and chitinase secretion, the optimal pH was found at the neutral pH ranges. Maximal lipolytic activity was observed at pH 7 (95.03 U/ml crude extract), while the highest secretion of chitinase was obtained at pH 6 (80.25 U/ml crude extract) ($p < 0.05$). Similar to protease secretion, both enzymes were secreted at very low level when cultivated at very acidic or alkaline pHs. The reduced activity was corresponding with the decreased growth. Normally, pHs of *Kapi* during production were in the neutral pH ranges (6.88-7.24) as reported by Pongsetkul *et al.* (2017b). Therefore, the optimal growth of *Bacillus* spp. K-C3 and the highest extracellular enzyme secretion was in the pH range of 6-8. It was suggested that this strain could survive and produce some enzymes when inoculated into *Kapi.*

12.5.2.2 Effect of temperature

Effect of temperature on the growth of *Bacillus* spp. K-C3 and the secretion of protease, lipase and chitinase was determined over temperature range of

25-60°C (Fig. 33B). The optimal growth of *Bacillus* spp. K-C3 was obtained when incubated at 35°C, followed by 30°C. The decrease in bacterial growth was observed as the incubation temperature increased. At 45°C, the growth was decreased more than 50%, compared to that of optimal temperature. This indicated that *Bacillus* spp. K-C3 could not grow well at the temperature higher than 45°C. All extracellular enzymes secreted by this *Bacillus* strain were obtained at the high level in temperature range of 25-40°C. However the optimum temperatures of each enzymes were different. *Bacillus* spp. K-C3 exhibited the highest protease secretion at 35°C as indicated by the maximum proteolytic activity of 148.83 U/ml crude extract ($p < 0.05$). The optimum temperature for lipase secretion was 25°C (85.63 U/ml crude extract), while the highest chitinase secretion was obtained at 30 and 35°C (75.42 and 75.36 U/ml crude extract, respectively) ($p < 0.05$). At the temperature higher than 45°C, the secretion of all enzymes were obviously decreased, which was in accordance with the decreased bacteria growth. Due to the lower cell growth at high temperature, the enzymes were produced to lower extent. The optimal pH and temperature of *Bacillus* spp. K-C3 were similar to those reported for other *Bacillus* species. *Alkalophilic Bacillus* sp. BG-11 had the optimal growth at the pH and temperature of 7.5 and 45°C, respectively (Bhushan and Hoondal, 1998). Maximal growth and protease production of *Bacillus* sp., Ve1 were obtained at pH 8 and 37°C (Patel *et al.,* 2005). Since the optimal temperature for growth and enzyme secretion of *Bacillus* spp. K-C3 was round 25-40°C, the secretion of extracellular enzymes after addition of this strain into *Kapi* could be possible. These enzymes were still active and improved *Kapi* characteristics by increasing hydrolysis or degradation of protein and lipid components.

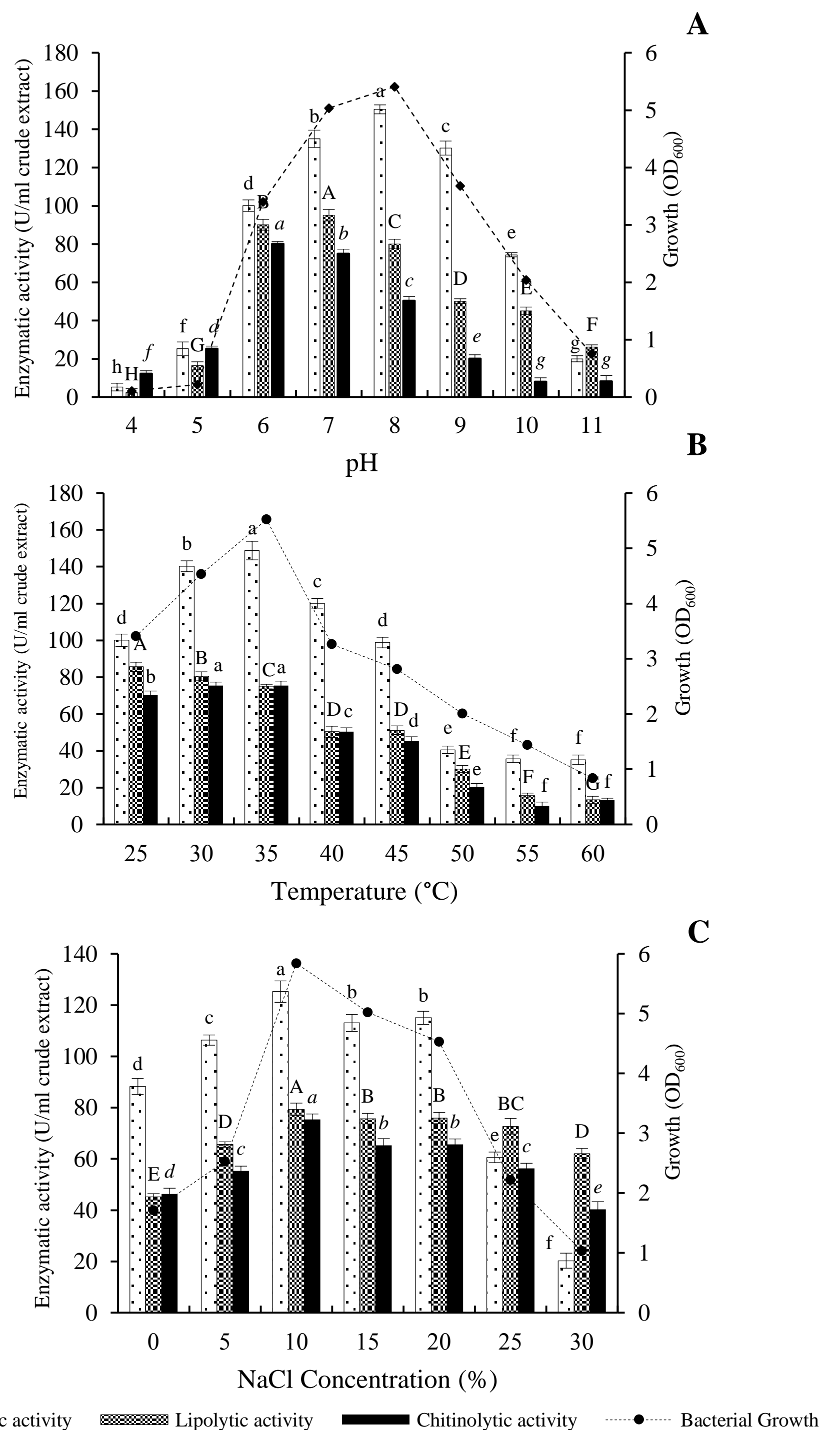

Figure 33. Effect of pH (A), temperature (B) and NaCl concentration (C) on growth and extracellular enzymes of *Bacillus* sp. K-C3.

Bars represent the standard deviation from triplicate determination. Different uppercase and lowercase letters indicate the significant differences ($p < 0.05$).

12.5.2.3 Effect of salt concentration

Effect of NaCl concentration on bacterial growth and their secretion of extracellular enzymes is shown in Figure 33C. At 10% NaCl, the highest bacterial growth was observed, followed by 15% and 20% NaCl, respectively ($p < 0.05$). The effect of salt concentration on the secretion of all enzymes was in agreement with the bacterial growth. All proteases, lipases and chitinases secreted by this *Bacillus* strain were observed at the highest level in the presence of 10% NaCl. The growth of *Bacillus* spp. K-C3 was drastically increased in the presence of 10% NaCl. Salt was a prerequisite for bacterial growth. When salt concentration was higher than 25% (w/v), the drastic decrease in bacterial growth was observed with coincidental decreases in protease and chitinase secretion. Nevertheless, the secretion of lipases was still high in the presence of high salt (25 and 30% (w/v)). Normally, salt content in commercial *Kapi* was in the range of 22.77-35.47% (Pongsetkul *et al.,* 2014). However, this bacterium was also able to grow and produce all extracellular enzymes at this salt level to some degrees.

12.5.3 Changes in *Bacillus* spp. K-C3 and enzyme activities of shrimp during *Kapi* production

12.5.3.1 Monitoring *Bacillus* spp. K-C3 using DGGE analysis

The hypervariable V3 region of 16S rDNA using primer 341f (GC) and 518R combined with DGGE analysis was applied for monitoring *Bacillus* spp. K-C3 during *Kapi* fermentation (Figure 34). Focusing on the existence of *Bacillus* spp. K-C3, there were no bands of this *Bacillus* strain found in fresh shrimp (lane 2) and shrimp after salting (lane 3). However, the specific band of *Bacillus* spp. K-C3 (lane 1) was observed in shrimp after drying process (lane 4). It was indicated that the sample was contaminated by this strain during drying with sunlight. Normally, *Bacillus* sp. is a gram-positive, facultative anaerobe bacterium found in environments, especially soil and air. It can form endospore to survive under the extreme environmental condition such as high temperature, low or high pH and desiccation (Nakano and Zuber, 1998). For the control sample (without inoculum), the specific

bands of *Bacillus* spp. K-C3 were observed and still remained throughout fermentation period up to 30 day. This was confirmed that this strain existed in the natural fermentation of *Kapi* and can survive during *Kapi* production with high salt concentration (20-30%, w/v), temperature (25-40°C) as well as anaerobic condition during fermentation. Similar to the control, the *Bacillus* spp. K-C3 bands were also detected in *Kapi* inoculated with starter culture at all levels and remained throughout fermentation process.

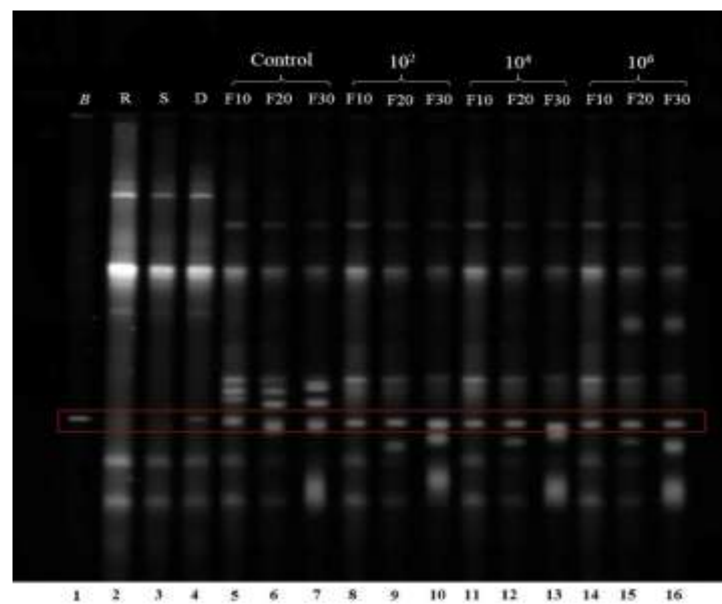

Figure 34. A DGGE profiles of shrimp during *Kapi* production. Lane 1, *Bacillus* spp. K-C3; Lanes 2-4 represented fresh shrimp (R) and shrimp after salting (S) and drying (D), respectively; Lanes 5-7 represented shrimp without inoculum (control) during fermentation for 10, 20 and 30 days, respectively. Lanes 8-10, 11-13, 14-16 represented shrimp inoculated with *Bacillus* spp. K-C3 at 10^2, 10^4 and 10^6 CFU/g sample during fermentation for 10, 20 and 30 days, respectively.

All inoculated samples contained similar band patterns at all fermentation periods tested. However, band patterns of inoculated *Kapi* samples were slightly different from the control (without inoculum). Inoculation of *Bacillus* strain could change overall microorganism populations in *Kapi* product and this could lead to differences in some final characteristics. With the conditions applied in the DGGE

protocol, the detection limit for individual members in the mixed populations was determined to be 10^4 CFU/g (data not shown). Therefore, when specific bands of *Bacillus* spp. K-C3 were detected, it only revealed the existence of this strain. Enumeration of this strain and other microorganisms need to be further detected by other analytical techniques.

12.5.3.2 Total viable count

TVC of *Kapi* inoculated without and with *Bacillus* spp. K-C3 during processing is presented in Table 35. Initial count of fresh shrimp used as raw material was 2.92 log CFU/g dry weight sample. Slight decrease in TVC after salting was observed, but TVC was increased after drying with the sunlight. When *Bacillus* spp. K-C3 at the levels of 10^2, 10^4 and 10^6 was inoculated before fermentation at room temperature for 1 month, TVC of all samples (both without and with inoculated) rapidly increased within the first 10 days of fermentation. The continuous increase of TVC was observed as fermentation time increased and reached 6.03-7.61 log CFU/g dry sample after 30 days of fermentation. During fermentation, proteolysis and lipolysis occurred. The products derived from those substrates including free amino acids, soluble non-nitrogenous substances as well as free fatty acids could be considered as available nutrients for microorganisms (Latorre-Moratalla *et al.,* 2011). All inoculated samples had the higher TVC at all steps of *Kapi* process, compared to the control ($p < 0.05$). The results also revealed that the amount of total microorganisms was related with the amount of starter culture inoculated into *Kapi.* Among all the inoculated samples, that inoculated with 10^6 CFU/g dry weight sample [*Kapi-B6*] contained the highest TVC throughout fermentation ($p < 0.05$). The resulted indicated that the higher amount of total microorganisms in the inoculated samples were belonging to starter culture added. *Bacillus* spp. K-C3 can grow well in the ranges of neutral pH to the slightly alkaline pH, room temperature as well as in the presence of high salt (Figure 33). From TVC result and DGGE analysis, it can be inferred that *Bacillus* spp. K-C3 could survive and still remained throughout *Kapi* processes, indicating the potential of *Bacillus* spp. K-C3 to be used as starter culture for *Kapi* production.

Table 35. Total viable count (TVC) of *Kapi* inoculated without and with *Bacillus* spp. K-C3 during fermentation

Samples	Total viable count (log CFU/g dry weight sample)
R	2.92±0.26^{A}
S	2.74±0.32^{B}
D	2.97±0.49^{A}
Day 10	
Kapi-C	4.36±0.13^{d}
Kapi-B2	5.62±0.54^{c}
Kapi-B4	6.28±0.26^{b}
Kapi-B6	7.23±0.60^{a}
Day 20	
Kapi-C	5.48±0.26^{c}
Kapi-B2	6.81±0.53^{b}
Kapi-B4	6.82±0.26^{b}
Kapi-B6	7.42±0.75^{a}
Day 30	
Kapi-C	6.03±0.34^{d}
Kapi-B2	7.04±0.36^{c}
Kapi-B4	7.20±0.12^{b}
Kapi-B6	7.61±0.46^{a}

R: raw material; S: after salting; D: after drying; *Kapi*-C: *Kapi* without inoculum, *Kapi*-B2, *Kapi*-B4 and *Kapi*-B6: *Kapi* inoculated with *Bacillus* spp. K-C3 at the level of 10^2, 10^4 and 10^6 CFU/ g dry weight sample, respectively.
Mean ± SD from triplicate determinations. Different uppercase superscripts of sample before fermentation indicate the significant differences ($p < 0.05$). Different lowercase superscripts within the same days of fermentation indicate the significant differences ($p < 0.05$).

12.5.3.3 Changes in enzyme activities of crude *Kapi* extract during *Kapi* production

During *Kapi* production, the changes in several enzyme activities including proteolytic, lipolytic as well as chitinolytic activities of the extract from *Kapi* inoculated without and with *Bacillus* spp. K-C3 at different levels were monitored (Figure 35). From Figure 35A, fresh shrimp had the proteolytic activity of 14.10 U/g dry weight sample, indicating the presence of endogenous proteases in the raw material and microflora proteases. After being salted, the activity was decreased to 12.87 U/g dry weight sample. This might be due to the inactivation of endogenous proteases by salt. Proteolytic activity was slightly increased after drying process, which was related

with the increase in TVC (Table 35). During sun-dried, some microorganisms in the air or environment could be contaminated and secreted the extracellular proteases to hydrolyze shrimp protein. Within the first 10 days of fermentation, the proteolytic activities of all samples were intensively increased. For *Kapi-C*, (without inoculated), proteolytic activity was rapidly increased and reached 23.39 U/g dry weight sample. Subsequently, the continuous increases were observed throughout fermentation process up to 30 days. Compared with the control, all inoculated *Kapi* samples had the higher proteolytic activity, especially during day 10-20 of fermentation. The result indicated that the higher proteolysis of shrimp proteins occurred in the inoculated samples. At day 10 of fermentation, *Kapi-B4* had the similar proteolytic activity (26.40 U/ g dry weight sample) to *Kapi-B6* (26.05 U/g dry weight sample). *Kapi-B2* had the lowest activity (25.61 U/g dry weight sample). Proteolytic activity of all inoculated *Kapi* samples was continuously increased up to 20 days, and then slightly decreased when fermentation time was prolonged. The decrease in proteolytic activity at the late stage of fermentation of all inoculated samples might be due to the denaturation and inhibition of the enzymes or reduction of substrate. Moreover, the loss in activity was plausibly due to the inhibition by end-products such as amino acid and short chain peptides (Klomklao *et al.,* 2006). The result clearly indicated that the inoculation with *Bacillus* spp. K-C3 directly affected proteolytic activity of *Kapi* during fermentation, particularly within the first 20 days of fermentation. Normally, hydrolysis of proteins in *Kapi*, is induced by endogenous enzymes in shrimp/krill as well as microbial enzymes surviving under high salt condition (Gildberg and Stenberg, 2001). Inoculation of *Bacillus* spp. K-C3, producing extracellular proteases, resulted in the increased proteolysis. This could accelerate the fermentation rate and affected the characteristics of final products. Gildberg and Thongthai (2001) reported that the addition of a halophilic lactic acid bacteria (*Tetragenococcus halophilus*), excreting some proteases, during low salt fish sauce production, could accelerate fermentation rate and also improved the organoleptic characteristics by increasing the tissue solubilization and flavor development of the product. Inoculation of *Pediococcus pentosaceus*, which had the strong proteolytic activity, in dried sausages resulted in the strong and rapid increases in short chain peptides and amino acid concentrations during

ripening. Nevertheless, those changes had no marked impact on the final flavor but accelerated maturation (Xu *et al.,* 2010).

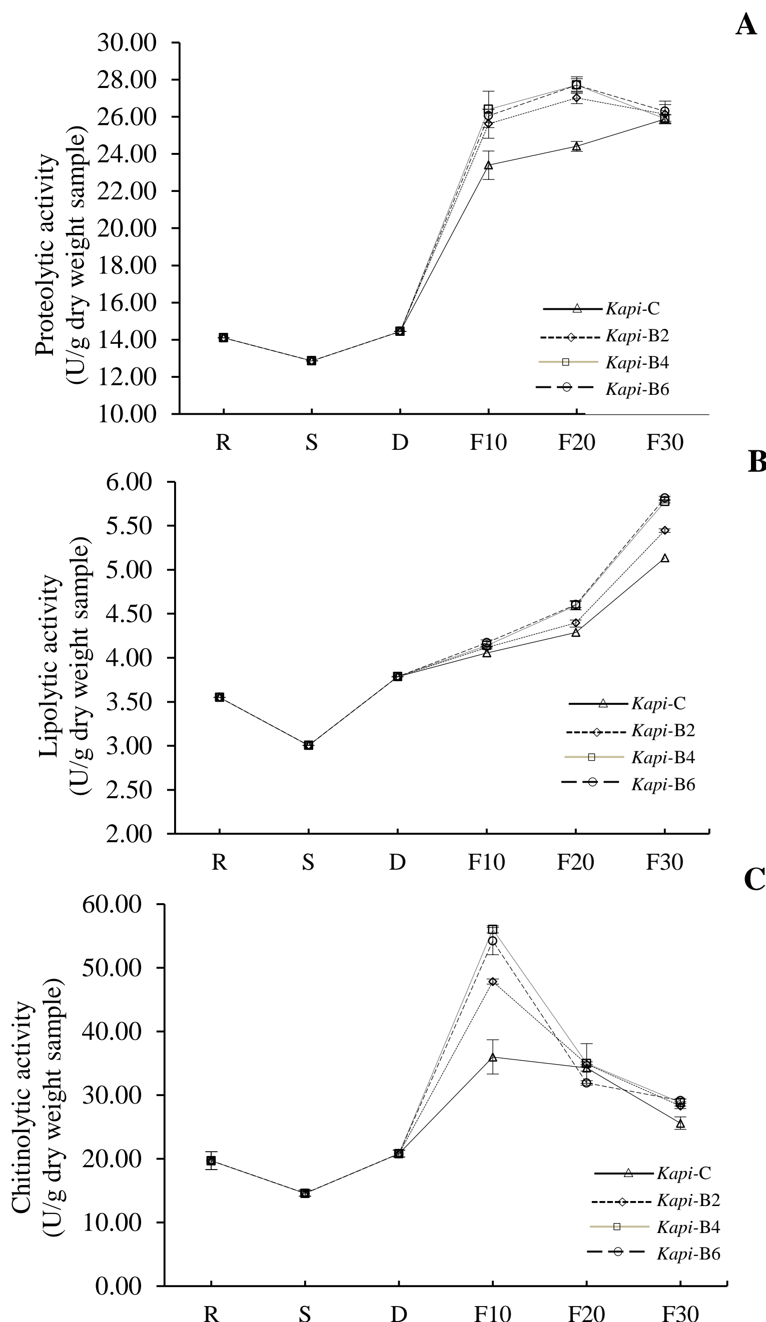

Figure 35. Proteolytic activity (A), lipolytic activity (B) and chitinolytic activity (C) of extracts from *Kapi* inoculated without and with *Bacillus* spp. K-C3 during *Kapi* production. R: raw material; S: after salting; D: after drying; F10, F20 and F30: after fermentation for 10, 20 and 30 days.

Bars represent the standard deviation from triplicate determination.

Lipolytic activity of shrimp inoculated without and with *Bacillus* spp. K-C3 during *Kapi* production is depicted in Figure 35B. Lipolytic activity of 3.55 U/g dry weight sample was found in fresh shrimp. Shrimp has been reported to contain a wide range of endogenous enzymes such as proteases (Senphan and Benjakul, 2014) and lipases (Kuepethkaew *et al.,* 2017). Hepatopancreas has been known to be the major source of enzymes in shrimp. The activity was decreased to 3.00 U/g dry weight sample after being salted. Slight decrease in lipolytic activity after salting might be due to the inactivation of endogenous lipases under high salt concentration. The increase in lipolytic activity was found after drying and the activity continuously increased during fermentation process in all samples, both without and with inoculum. Thus, the combination role of both remaining endogenous lipases in shrimp as well as microbial lipases secreted under the high salt condition were mainly involved in lipolysis. Marked increase in lipase activity of all samples was observed at the later stage of fermentation, particularly when fermentation time was more than 20 days. Among all the samples, *Kapi-B6* had the highest lipolytic activity at all fermentation periods, followed by *Kapi-B4* and *Kapi-B2,* respectively. This confirmed that *Bacillus* spp. K-C3, used as inoculum, play a profound role in lipolysis during *Kapi* fermentation. The higher lipolytic activity of inoculated samples could bring about the better final quality of *Kapi.* Many previous reports revealed that FFA concentration and some lipid oxidation products were associated with *Kapi* characteristics, particularly flavor and odor (Cha and Cadwallader, 1995).

Change in chitinolytic activity of shrimp inoculated without and with *Bacillus* spp. K-C3 during *Kapi* production was monitored (Figure 35C). Fresh shrimp had chitinolytic activity of 19.69 U/g dry weight sample. Similar to other enzyme activities, chitinolytic activity decreased to 14.58 U/g dry weight sample after salting overnight and then slightly increased after drying process up to 20.82 U/g dry weight sample. Subsequently, the sharp increase in chitinolytic activity was observed after fermentation. The highest chitinolytic activity during *Kapi*, both without and with inoculum, was found within the first 10 days of fermentation. Compared with the control, *Kapi* inoculated with *Bacillus* spp. K-C3 at all levels exhibited the higher chitinolytic activity. At day 10 of fermentation, *Kapi-B2* had the chitinolytic activity of 47.82 U/g dry weight sample, which was lower than those of *Kapi-B4* and *Kapi-B6.*

However, there was a slight difference in chitinolytic activity between *Kapi-B4* and *Kapi-B6*. *Bacillus* spp. K-C3 could therefore secrete extracellular chitinases. Chitinase secrered from the starter culture, in combination with chitinase from raw material or microflora in *Kapi,* still remained and were active under high salt condition. Thereafter, chitinase activity of all samples was decreased as fermentation time increased up to 30 days. Chitin is a major component of crustacean shells and is degraded by chitinase, which can be found in various bacteria, fungi, etc. Some bacteria produce chitinase to degrade chitin primarily to provide carbon and nitrogen as nutrients (Stephane *et al.,* 2005). High activity of chitinase at the first period of fermentation might be due to sufficient nutrients and suitable condition. The higher chitinase after inoculation might result in the higher rate of chitin degradation. This could affect the texture of final *Kapi,* in which softer or pastier texture was attained. The decrease in this activity at the later stage of fermentation might be due to the loss in enzyme activity.

Overall, shrimp *A. vulgaris*, using as raw material, contained some amount of endogenous proteases, lipases as well as chitinases. During *Kapi* production, especially fermentation period, these enzyme activities were drastically increased. These might be from the combination between endogenous enzymes, and those from halophilic bacteria survived under high salt condition. Inoculation with *Bacillus* spp. K-C3 at different levels could increase all enzyme activities during *Kapi* fermentation. The higher amount of those enzymes contributed to accelerated fermentation of *Kapi,* leading to the faster development of final characteristics with the preferable quality.

12.6 Conclusion

The growth and the production of extracellular enzymes including protease, lipase and chitinase of *Bacillus* spp. K-C3, isolated from commercial *Kapi,* had the optimal pH of 7-8 and optimal temperature of 25-35°C, with an incubation time of 24-48 h. Salt at the concentration of 10% (w/v) increased both bacterial growth and enzymatic production of this *Bacillus* strain. Inoculation with *Bacillus* spp. K-C3 before fermentation resulted in the higher fermentation rate as indicated by higher proteolytic,

lipolytic as well as chitinolytic activities. Therefore, *Bacillus* spp. K-C3, which could survive until the completion of *Kapi* production as indicated by DGGE analysis, might enhance the development of final characteristics and could shorten the fermentation time of *Kapi*. These results demonstrated the possibility of *Bacillus* spp. K-C3 as potential starter culture for *Kapi* production. The impact of this strain on final *Kapi* quality will be further investigated.

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CHAPTER 13

QUALITY OF *KAPI*, SALTED SHRIMP PASTE OF THAILAND, INOCULATED WITH *BACILLUS* SPP. K-C3

13.1 Abstract

The uses of *Bacillus* spp. K-C3, isolated from commercial *Kapi*, a salted shrimp paste of Thailand, as the starter culture for *Kapi* production, at different levels including 10^2, 10^4 and 10^6 CFU/ g dry weight sample named *Kapi-B2*, *Kapi-B4* and *Kapi-B6*, respectively, in comparison with naturally fermented *Kapi* (*Kapi-C*), were studied. All inoculated samples exhibited the higher extent of proteolysis and lipolysis as indicated by higher TCA-soluble peptide, degree of hydrolysis and free fatty acid content in the final products. The greater rate of fermentation in the inoculated samples directly affected characteristics of *Kapi* and yield the *Kapi* with browner color and enhanced lipid oxidation as indicated by PV and TBARS values. Increased antioxidative properties (DPPH, ABTS radical scavenging activities and FRAP) were obtained in *Kapi* added with inoculum. Moreover, those inoculated samples also contained the higher intensity of volatile compounds, in which *N*-containing compounds, mainly pyrazine derivatives, were dominant. *Kapi-B4* and *Kapi-B6* showed the highest extents of these aforementioned values, compared to *Kapi-C* and *Kapi-B2*. Therefore, inoculation with *Bacillus* spp. K-C3 at the level higher than 10^4 CFU/ g dry weight sample was the potential means to accelerate the fermentation rate and yielded *Kapi* with the preferable characteristics.

13.2 Introduction

Salted shrimp paste is one of fermented foods commonly used as the condiment with salty and umami taste in many Southeast-Asian countries (Hajeb and Jinap, 2015). Traditionally, salted shrimp paste is made by mixing ground krill (*Mesopodopsis* spp.) or shrimp (*Acetes* spp.) with the solar salt. The mixture is dried under the sunlight, then ground into fine paste and allowed to ferment at room temperature at least 1 month or until the typical aroma is developed (Pongsetkul *et al.,*

2017a). Salted shrimp paste is known in various names among different countries, for example, *Belacan* in Malaysia, *Terasi* in Indonesia, *Bagoong-alamung* in the Philippines, *Mamruoc* or *Mamtom* in Vietnam, *Ngapi* in Burma or *Nappi* in Bangladesh, etc (Hajeb and Jinap, 2015).

In Thailand, the traditional salted shrimp paste, known as *Kapi*, has various colors from purplish-pink to dark-brown. The consistency of *Kapi* also varies from soft and pasty to dry and hard (Faithong *et al.,* 2010). Normally, the different characteristics and properties of *Kapi* are governed by various manufacturing processes and factors such as the type of raw material, the amount of salt used, the fermentation conditions as well as the presence of different naturally occurring bacteria in the product (Phitakpol, 1993; Faithong *et al.,* 2010; Pongsetkul *et al.,* 2015). These differences could determine the quality of the product or consumer's acceptance.

The use of starter cultures in meat/seafood fermentation products has become well established recently as a means to increase processing rates and product consistency. Additionally, starter culture can improve the sensory characteristics and microbiological quality of products (Visessanguan *et al.,* 2006). The selection of starter cultures is evaluated by both ability of isolates to ferment food substances and the sensory quality of the final product (Visessanguan *et al.,* 2006). In general, proteolysis and lipolysis are the important biochemical changes occurring during *Kapi* production, especially during fermentation process. These changes are induced by endogenous enzymes in shrimp/krill as well as microbial enzymes surviving under high salt condition (Pongsetkul *et al.,* 2017a). Products from hydrolysis of proteins and lipids such as peptides, amino acids, free amino or fatty acids, etc. are the main precursors for the development of several volatile compounds, which contribute to odor and flavor of the *Kapi* (Itou *et al.,* 2006; Wittanalai *et al.,* 2011; Pongsetkul *et al.,* 2017a).

Bacillus sp. have the potential starter culture due to their ability to hydrolyze proteins and lipids (Surono and Hosono, 1994). From our previous study, *Bacillus* spp. K-C3 from commercial *Kapi* has been isolated. This strain was able to produce a wide range of extracellular enzymes including protease, lipase as well as chitinase. Those enzymes could accelerate the degradation of protein, lipid and chitin. Therefore, inoculation of *Bacillus* spp. K-C3 might be used to shorten the processing

time and to improve the quality of final *Kapi*, especially the increased development of aroma volatile compounds.

13.3 Objective

To evaluate the chemical and physical characteristics of *Kapi* produced by inoculation with *Bacillus* spp. K-C3, isolated from commercial *Kapi*, at different levels, compared to that with natural fermentation (*Kapi-C*) (without inoculum).

13.4 Materials and methods

13.4.1 Preparation of starter cultures

Bacillus sp. K-C3 was isolated from commercial *Kapi* and kept as lyophilized culture at -80°C. The *Bacillus* culture was subcultured by transferring a loopful of microorganisms into 50 ml of sterile nutrient broth. The culture was incubated at 37.5°C for 24 h in a rotary shaker with an agitation rate of 180 rpm. Then, cells were then harvested by centrifugation at 10,000×*g* for 5 min at 4°C using the refrigerated centrifuge (Beckman Coulter, Avanti J-E Centrifuge, Palo Alto, CA, USA). Thereafter, cells were washed with 0.85% (w/v) saline water (NaCl) for 3 times. Finally, the dilution of microorganisms was made in sterile 0.85% (w/w) NaCl solution to obtain 1×10^8 colony forming unit (CFU)/ml and used as the inoculum for fermentation of *Kapi*.

13.4.2 Preparation of *Kapi*

Shrimp *Acetes vulgaris* (average body length 15.6±1.4 mm, average wet weight 0.0413±0.0098 g, n=20) were obtained and transported in ice from the local market in Ko-yo, Songkhla, Thailand to the laboratory within 1 h. Upon arrival, 10 kg of shrimp were washed twice with distilled water containing 3% NaCl (w/v). Then, solar salt was added at the shrimp/salt ratio of 5:1 (w/w) and mixed well. The mixture was allowed to stand at room temperature overnight, followed by draining. The sample was ground to uniformity using a blender (National, Tokyo, Japan). Then, salted shrimp were dried under the sunlight until the moisture content was decreased to the range of

35-40% (AOAC, 2000). Subsequently, the inoculum was added to obtain the final cell concentrations of 10^2, 10^4 and 10^6 CFU/g dry weight sample, followed by thorough mixing. The prepared samples were impacted into earthen jars, covered with the cheese cloth and fermented at room temperature (28-30°C) for 30 days. The control was prepared in the same manner but the inoculum was excluded.

13.4.3 Characterization of *Kapi* quality inoculated with *Bacillus* spp. K-C3 at different levels

13.4.3.1 Color

Color of samples was determined using a colorimeter (ColourFlex, Hunter Lab Reston, VA, USA) and reported in the CIE system. L^* (lightness), a^* (redness/greenness) and b^* (yellowness/blueness) were recorded. Additionally, ΔE^* (total difference of color) and ΔC^* (the difference in chroma) of the inoculated *Kapi* samples were calculated as described by Pongsetkul *et al.* (2017a).

13.4.3.2 Browning intensity

Kapi samples were firstly subjected to extraction using distilled water as medium according to the method of Pongsetkul *et al.* (2017a). *Kapi* (2 g) was mixed with 50 ml of distilled water. The mixtures were homogenized at a speed of 10,000×*g* for 2 min using an IKA Labortechnik homogenizer (Selangor, Malaysia). The homogenates were then centrifuged at 13,000×*g* for 15 min at room temperature. The supernatant was collected. Appropriate dilution was made using distilled water and the absorbance was read at 420 nm using the UV-1601 spectrometer (Shimadzu, Kyoto, Japan).

13.4.3.3 TCA-soluble peptide content

TCA-soluble peptide contents of *Kapi* samples were determined after precipitation with 5% (w/v) TCA. TCA-soluble peptide content was measured according to the Lowry method (Lowry *et al.,* 1951) and expressed as mmol tyrosine equivalent/kg dry weight sample.

13.4.3.4 Degree of hydrolysis (DH)

DH of *Kapi* was determined according to the method of Benjakul and Morrissey (1997). One gram of *Kapi* samples was mixed with 9 ml of 5% (w/v) SDS. The mixture was homogenized at a speed of 11,000 rpm for 1 min. The homogenate was heated at 85°C for 30 min and then subjected to centrifugation at 10,000×*g* for 15 min at room temperature. The supernatant was analyzed. The DH of samples was calculated and expressed as percentage.

13.4.3.5 Free fatty acid (FFA) content

Lipids were extracted from 25 g of each *Kapi* samples with a solvent mixture of chloroform-methanol-distilled water (50:100:50, v/v) according to the method of Bligh and Dyer (1959). FFA content of lipid samples was determined according to the method of Lowry and Tinsley (1976). A standard curve was prepared using palmitic acid in isooctane at concentrations ranging from 0 to 10 mM. FFA content was expressed as g FFA/ 100 g lipid.

13.4.3.6 Peroxide value (PV)

PV was measured following the method of Takeungwongtrakul and Benjakul (2013). A standard curve was prepared using cumene hydroperoxide at the concentration range of 0.5-2 ppm. PV was reported as mg hydropreoxide/kg dry weight sample.

13.4.3.7 Thiobarbituric acid reactive substances (TBARS) value

TBARS value was determined as per the method of Takeungwongtrakul and Benjakul (2013). A standard curve was prepared using malonaldehyde bis (dimethyl acetal) at concentrations ranging from 0 to 2 ppm. TBARS value was expressed as mg malonaldehyde (MDA)/kg dry weight sample.

13.4.3.8 Antioxidative activities

Water extracts of all samples prepared as mentioned above were determined for antioxidative activities. Prior to assay, the extracts were approximately diluted using distilled water. 2,2-diphenyl-1-picrylhydrazyl (DPPH) and 2,2-azino-bis (3-ethylbenzthiazoline-6-sulphonic acid) (ABTS) radical scavenging activities, as well as ferric reducing antioxidant power (FRAP) were determined according to the method of Faithong *et al.* (2010). Activities were expressed as μmol Trolox equivalents (TE)/g sample.

13.4.3.9 Volatile compounds

Volatile compounds of the samples were determined using a solid-phase microextraction gas chromatography mass spectrometry (SPME GC-MS) as per the method of Pongsetkul *et al.* (2015). Volatile compounds were identified and expressed in the terms of relative abundance.

13.4.4 Statistical analysis

All experiments were run in triplicate using three different lots of samples. Statistical analysis was done by using one-way analysis of variance (ANOVA) according to the method of Steel *et al.* (1980). The statistical differences of all data were analyzed by the Duncan's multiple range test using the SPSS package version 10.0 (SPSS for window, SPSS Inc., Chicago, IL, USA). Statistical significance was considered at P value less than 0.05.

13.5 Results and discussion

13.5.1 Color

Kapi samples inoculated with *Bacillus* sp. K-C3 at various levels had different color characteristics (Table 36). Among all samples, *Kapi-B4* had the lowest L^*-value but the highest a^*- and ΔE^*-values ($p < 0.05$). This sample therefore exhibited more redness and browner than other samples ($p < 0.05$). Generally, the color of *Kapi* was developed gradually as the fermentation time increased. The decreases in L^*- and

b^*-values but the increase in a^*-value were observed as the fermentation time increased (Pongsetkul *et al.,* 2017a). *Kapi* inoculated with starter culture at all levels had the lower L^* and b^*-values but showed the higher a^*-value, compared to the control (without inoculum). The highest ΔC^*-value but lowest ΔE^*-value were noticeable in the control *Kapi* (without inoculum). This indicated that color changes during *Kapi* processing of the inoculated samples were more pronounced than that of the control. Inoculation of starter culture could accelerate the color development of *Kapi* product, especially brown color. Our previous study revealed that *Bacillus* sp. K-C3 produced several many extracellular enzymes including proteases and lipases (data not shown). These enzymes secreted were more likely retained throughout *Kapi* processing. During fermentation, those microbial enzymes as well as indigenous enzymes were able to hydrolyze carotenoproteins. As a consequence, free carotenoids, particularly astaxanthin, were liberated, resulting in more reddish color of the inoculated samples. Astaxanthin is a pigment commonly found in crustacean, providing the tissue with red-orange pigmentation (Higuera-Ciapara *et al.,* 2006). Therefore, the inoculation with *Bacillus* sp. K-C3 in the partially dried shrimp before subjected to fermentation could enhance the fermentation via increasing protein and lipid degradation/decomposition. This was associated with the higher development of color in the inoculated *Kapi,* especially the brown color.

Table 36. Color of *Kapi* inoculated without and with *Bacillus* spp. K-C3 at different levels

Sample	Color				
	L^*	a^*	b^*	ΔE^*	ΔC^*
Kapi-C	39.98 ± 1.54^a	8.46 ± 0.13^c	22.99 ± 0.53^a	58.89 ± 1.60^d	24.50 ± 0.53^a
Kapi-B2	36.74 ± 0.49^b	8.53 ± 0.22^b	21.93 ± 0.12^b	61.48 ± 0.50^b	23.53 ± 0.13^b
Kapi-B4	35.01 ± 0.62^c	8.61 ± 0.17^a	20.94 ± 0.10^c	62.76 ± 0.54^a	22.64 ± 0.10^c
Kapi-B6	36.89 ± 1.01^b	8.52 ± 0.04^b	20.70 ± 0.16^c	60.92 ± 0.98^c	22.38 ± 0.14^c

Kapi-C: *Kapi* without inoculum, *Kapi*-B2, *Kapi*-B4 and *Kapi*-B6: *Kapi* inoculated with *Bacillus* spp. K-C3 at the level of 10^2, 10^4 and 10^6 CFU/ g dry weight sample, respectively.
Mean ± SD from triplicate determinations. Different lowercase superscripts in the same column indicate the significant difference ($p < 0.05$).

13.5.2 Browning intensity

Browning intensity (A_{420}) of water extracts from *Kapi* samples inoculated without and with *Bacillus* spp. K-C3 at different levels is shown in Table 37. In general, A_{420} was used as an indicator for browning development in the final stage of browning reaction (Benjakul *et al.,* 1997). Among all samples, *Kapi-B4* had the highest browning intensity (0.58). Moreover, higher browning intensity was found in all inoculated samples, compared with the control. This was generally in agreement with the lower *L**- and *b**-values, along with the higher *a**-value of the inoculated samples. Processing or fermentation process directly influenced the brown color development of *Kapi* via Maillard reactions (Pongsetkul *et al.,* 2017a). The Maillard reaction comprises the condensation between a carbonyl group of reducing sugars, aldehydes or ketones and an amino group of free amino acids (such as amino acids, peptides and proteins) or any nitrogenous compound (Jing and Kitts, 2004). During *Kapi* fermentation, most of nitrogenous compounds including free amino acids and small peptides were generated and acted as reactants in Maillard reaction (Lopetcharat *et al.,* 2001). Carbohydrate derivatives, such as glucose-6-phosphate and other substances present in the metabolic pathways, could also act as reactants in the Maillard reaction (Jing and Kitts, 2004). Moreover, oxidation products, such as aldehyde were able to react with free amino acids, liberated during fermentation. These might cause the browner color of *Kapi* when fermentation proceeded. The result indicated that the addition of *Bacillus* spp. K-C3, especially at the level of 10^4 CFU/g dry weight sample, could accelerate the brown color development of the final product. Protease or lipases secreted by the starter culture added might increase the reactants for browning reaction. Generally, the color of *Kapi* is the one factor affecting the overall consumer acceptance and the brown color is a preferable quality attribute of *Kapi* according to the Thai Industrial Standard for this product (1988).

Table 37. Browning intensity (A_{420}) and antioxidative properties of water extracts from *Kapi* inoculated without and with B*acillus* spp. K-C3 at different levels

Samples	Browning intensity (A_{420})	DPPH radical scavenging activity*	ABTS radical Scavenging activity*	FRAP*
Kapi-C	$0.42{\pm}0.03^{c}$	$6.79{\pm}0.18^{b}$	$13.88{\pm}0.22^{c}$	$15.77{\pm}0.16^{b}$
Kapi-B2	$0.45{\pm}0.08^{b}$	$8.77{\pm}0.26^{a}$	$13.95{\pm}0.51^{c}$	$15.36{\pm}0.02^{c}$
Kapi-B4	$0.53{\pm}0.02^{a}$	$8.53{\pm}0.21^{a}$	$14.64{\pm}0.13^{b}$	$15.81{\pm}0.45^{b}$
Kapi-B6	$0.46{\pm}0.05^{b}$	$8.91{\pm}0.37^{a}$	$15.22{\pm}0.27^{a}$	$16.18{\pm}0.58^{a}$

*Expressed as μmol TE/g sample.
Kapi-C: *Kapi* without inoculum, *Kapi*-B2, *Kapi*-B4 and *Kapi*-B6: *Kapi* inoculated with *Bacillus* spp. K-C3 at the level of 10^2, 10^4 and 10^6 CFU/ g dry weight sample, respectively.
Mean ± SD from triplicate determinations. Different lowercase superscripts in the same column indicate the significant difference ($p < 0.05$).

13.5.3 TCA-soluble peptide content

Figure. 36A showed TCA-soluble peptide content of different *Kapi* samples. TCA-soluble peptide content indicates proteolytic degradation occurring during *Kapi* production. *Kapi-C* had the lowest TCA-soluble peptide content (0.57 mmol/kg dry weight sample) ($p < 0.05$). All *Kapi* samples inoculated with *Bacillus* spp. K-C3 had the higher TCA-soluble peptide content, compared with the control, indicating the higher proteolysis of shrimp proteins of inoculated samples. The results also revealed that the extent of proteolysis was related with the amount of starter culture inoculated into *Kapi.* Proteases produced from *Bacillus* spp. K-C3 played a role in hydrolysis of shrimp proteins to oligopeptides or free amino acids. Therefore, the inoculation with *Bacillus* spp. K-C3 in *Kapi* resulted in an increased proteolysis, which might be associated with the development of *Kapi* characteristics.

13.5.4 Degree of hydrolysis

DH of each *Kapi* samples without and with inoculum at various levels is shown in Figure 36B. DH is the measure of the extent of cleavage of peptide linkages. DH close to 100 % means that all proteins in the sample are completely hydrolyzed to free amino acids (Benjakul *et al.,* 1997). Among all *Kapi* samples, *Kapi-C* had the lowest DH (23.51%). All inoculated samples had the higher DH, compared to *Kapi-C*,

but there was no difference among all inoculated samples ($p > 0.05$). The differences in protein degradation between *Kapi-C* and inoculated samples were mostly due to the extracellular proteases from *Bacillus* spp. K-C3, used as starter culture. *Bacillus* proteases, in combination with endogenous proteases from shrimp, could hydrolyze shrimp proteins into smaller peptides or free amino acids. Overall, proteolysis of shrimp proteins inoculated with *Bacillus* spp. K-C3 occurred at the higher degree than the control. The differences in proteolysis might contribute to the different taste and aroma of the final *Kapi.*

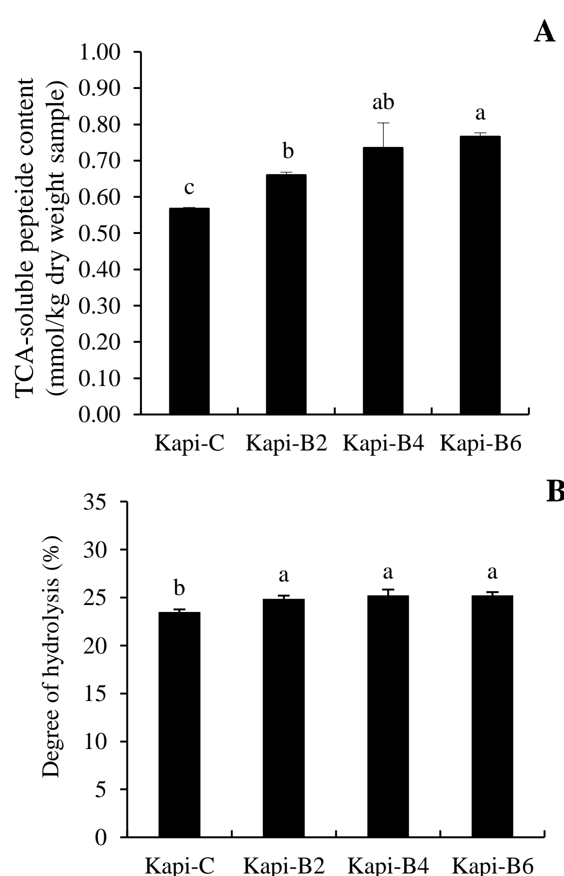

Figure 36. TCA-soluble peptide content (A) and degree of hydrolysis (B) of *Kapi* inoculated without and with *Bacillus* spp. K-C3 at different levels. *Kapi-C*: *Kapi* without inoculum, *Kapi-B2*, *Kapi-B4* and *Kapi-B6*: *Kapi* inoculated with *Bacillus* spp. K-C3 at the level of 10^2, 10^4 and 10^6 CFU/g dry weight sample, respectively.

Bars represent the standard deviation from triplicate determinations. Different letters on the bars denote the significant differences ($p < 0.05$).

13.5.5 Free fatty acid (FFA) content

Free fatty acid (FFA) contents of *Kapi* inoculated without and with *Bacillus* spp. K-C3 at different levels are depicted in Figure 37A. The lowest FFA content was found in *Kapi-C* sample (5.20 g/100 g lipid sample) ($p < 0.05$). Compared with the control, all inoculated *Kapi* samples contained the higher FFA contents. *Kapi-B2* had the FFA content of 5.65 g/100 g lipid sample, which was lower than those of *Kapi-B4* and *Kapi-B6* ($p < 0.05$). However, there was no difference in FFA content between *Kapi-B4* and *Kapi-B6* ($p > 0.05$). Lipolysis occurring during *Kapi* fermentation was attributed to both lipases of the muscular tissue of shrimp and microbial origin (Pacheco-Aguilar *et al.,* 2000). In general, lipolysis is the one of biochemical changes taken place throughout *Kapi* processing, particularly during fermentation (Pongsetkul *et al.,* 2017b). Hydrolysis of glycerol-fatty acid esters is one important change found in fish/shrimp muscle lipids during post-mortem handling or processing. This is catalyzed by lipases and phospholipases (Pacheco-Aguilar *et al.,* 2000). The accumulation of FFA in the final *Kapi* product could be mediated by lipase and phospholipase in shrimp muscle, digestive organs as well as microorganisms. The result suggested that inoculation with *Bacillus* spp. K-C3 could enhance lipolysis of *Kapi*. Inoculation with *Bacillus* spp. K-C3 at the higher level results in the higher degradation of shrimp lipids during *Kapi* fermentation.

13.5.6 Lipid oxidation products

Peroxide value (PV) and TBARS value of *Kapi* inoculated without and with *Bacillus* spp. K-C3 at various levels are shown in Figure 37B and 37C, respectively. Generally, lipid oxidation products had impact on sensory characteristics of many fermented foods. Cha and Cadwallader (1995) showed that the oxidation of lipids accounted for about 60% of the total compounds responsible for fish/shrimp paste flavor. However, an excessive lipid oxidation products can limit shelf-life of products. PV measures the formation of peroxide or hydroperoxide groups that are the initial products of lipid oxidation, while TBARS have been used to measure the concentration of relatively polar secondary reaction products, especially aldehydes (Takeungwongtrakul and Benjakul, 2013). Both PV and TBARS values were used as

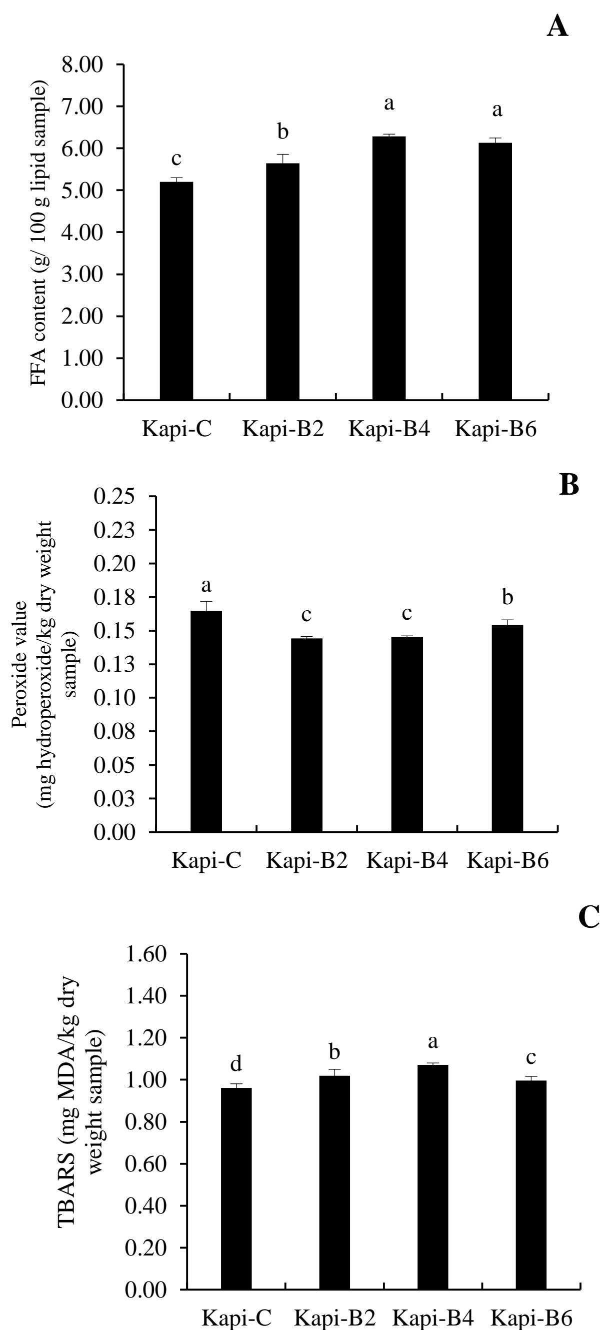

Figure 37. FFA content (A), Peroxide value (B) and TBARS value (C) of *Kapi* inoculated without and with *Bacillus* spp. K-C3 at different levels. *Kapi-C*: *Kapi* without inoculum, *Kapi-B2*, *Kapi-B4* and *Kapi-B6*: *Kapi* inoculated with *Bacillus* spp. K-C3 at the level of 10^2, 10^4 and 10^6 CFU/g dry weight sample, respectively.

Bars represent the standard deviation from triplicate determinations. Different letters on the bars denote the significant differences ($p < 0.05$).

indices to assess the level of lipid oxidation occurring in *Kapi* samples. The highest PV was found in *Kapi-C* (0.16 mg hydroperoxide/g dry weight sample), while *Kapi-B4* and *Kapi-B2* had the lowest PV (0.146 and 0.144 mg hydroperoxide/g dry weight sample, respectively) ($p < 0.05$). Lipid hydroperoxides are formed by various pathways including the reaction of singlet oxygen with unsaturated lipids or the lipoxygenase-catalyzed oxidation of PUFA (Boselli *et al.,* 2005). Shrimp has been reported to contain high amount of PUFA (Takeungwongtrakul and Benjakul, 2013). Therefore, lipid oxidation in *Kapi* was owing to the high content of unsaturated fatty acids of shrimp used as raw material. The decrease in PV was noticeable when fermentation proceeded due to the decomposition of hydroperoxide to the secondary oxidation products (Boselli *et al.,* 2005) as evidenced by the increase in TBARS value. *Kapi-C,* which showed the highest PV, had the lowest TBARS value (0.96 mg MDA/kg dry weight sample). All inoculated samples possessed the higher TBARS value, compared to *Kapi-C* ($p < 0.05$), indicating that the lipid oxidation proceeded to the higher extent when inoculated with *Bacillus* spp. K-C3. During fermentation, the disruption of the organelles associated with the release of pro-oxidants as well as reactants took place (Takeungwongtrakul and Benjakul, 2013). This resulted in the enhanced lipid oxidation in *Kapi.* Some lipid oxidation products might contribute to typical odor and flavor of *Kapi.* The different lipid hydrolysis and oxidation could also lead to differences in quality or acceptability of the final products.

13.5.7 Antioxidant activities

Antioxidative activities of water extracts of different *Kapi* samples as examined by DPPH, ABTS radical scavenging activities and FRAP are shown in Table 37. Among all samples, *Kapi-C* showed the lower DPPH radical scavenging activity (6.79 μmol TE/g dry weight sample) than those of all inoculated samples ($p < 0.05$). DPPH radical is considered to be a model of the lipophilic radical. Lipophilic radicals are initiated by lipid autoxidation (Benjakul *et al.,* 2005). The higher DPPH radical scavenging activity of inoculated *Kapi* samples indicated the higher ability in donating hydrogen atom to free radicals, in which the propagation process of oxidation could be retarded. This might be due to higher small peptides generated from proteolysis in the inoculated samples as indicated by the higher TCA-soluble peptide content (Figure

36A) and DH (Figure 36B). Those small peptides could act as antioxidants capable of donating hydrogen atom. Degree of hydrolysis directly affects the antioxidative activity of protein hydrolysate (Wu *et al.,* 2003). Moreover, the inoculated samples mostly contained the higher Maillard reaction products (MRPs) as indicated by the higher browning intensity (Table 37). MRPs have been found to exhibit antioxidative activity due to radical scavenging activity, metal chelating activity and scavenging of active oxygen species (Benjakul *et al.,* 2005). The highest ABTS radical scavenging activity was found in *Kapi-B6* (15.22 μmol TE/g dry weight sample), followed by *Kapi-B4* (14.64 μmol TE/g dry weight sample). However, there was no difference between *Kapi-B2* and the control ($p > 0.05$). In general, ABTS assay is an excellent tool for determining both hydrophilic and lipophilic antioxidants (Wu *et al.,* 2003). Similarly, the inoculation with *Bacillus* spp. K-C3 could increase ABTS radical scavenging activity of *Kapi.* For FRAP activity, the highest activity was also noticeable in *Kapi-B6* (16.18 μmol TE/g dry weight sample), while *Kapi-B2* had the lowest FRAP activity (15.36 μmol TE/g dry weight sample). In general, FRAP is generally used to measure the capacity of a substance in reducing TPTZ-Fe(III) complex to TPTZ-Fe(II) complex (Faithong *et al.,* 2010). The result indicated the different capacity of providing the electron among various *Kapi* samples.

Overall, all *Kapi* samples possessed antioxidative activities. Nevertheless, inoculation with *Bacillus* spp. K-C3 at the level of 10^6 CFU/g dry weight sample could increase the antioxidative properties of the final *Kapi* product as tested by all assays. Higher protein degradation during production of *Kapi* with inoculum led to the generation of low molecular weight peptides and amino acids, which were able to terminate the chain reaction of auto-oxidation.

13.5.8 Volatile compounds

Volatile compounds of *Kapi* inoculated without and with *Bacillus* spp. K-C3 at different levels and fresh shrimp (raw material) were identified (Table 38). The major typical volatile compounds found in all *Kapi* samples were divided into seven main groups, which were *N*-containing compounds are the most prominent volatiles found in fresh shrimp and all *Kapi* products, accounting for more than 40% of total abundance.

Table 38. Volatile compounds of *Kapi* inoculated without and with *Bacillus* spp. K-C3 at different levels

Volatile compounds	Peak area (Abundance)×10^6				
	Fresh shrimp	*Kapi*-C	*Kapi*-B2	*Kapi*-B4	*Kapi*-B6
Aldehydes					
2-ethyl-butanal	ND	ND	99.65	62.63	35.11
2-methyl-butanal	10.00	14.43	54.54	222.55	142.67
3-methyl-butanal	5.04	203.22	405.31	402.11	503.29
Pentanal	ND	17.76	82.22	52.67	42.18
2-hexanal	13.81	9.22	60.07	18.13	22.33
Heptanal	ND	16.16	14.23	44.32	46.46
2-octenal	7.92	2.03	4.05	ND	ND
Benzaldehyde	105.66	99.98	111.25	206.62	155.54
Total aldehydes	**142.43**	**362.80**	**831.32**	**1009.03**	**957.58**
Ketones					
1-phenyl-ethanone	ND	47.23	70.02	64.91	24.43
1,2-diphenyl-ethanone	18.22	ND	8.55	9.33	ND
1-(2-aminophenyl)-ethanone	ND	22.25	46.63	22.53	9.98
2-pentanone	ND	8.33	5.01	27.61	13.55
2-hexanone	86.09	52.92	92.66	255.06	286.77
1-phenyl-1-hexanone	ND	ND	55.52	56.44	42.39
2-heptanone	22.30	42.43	4.01	ND	6.02
6-methyl-5-hepten-2-one	ND	ND	2.61	ND	ND
2-octanone	ND	ND	ND	22.66	19.08
3-octanone	102.24	56.66	14.44	8.11	ND
3,5-octadiene-2-one	99.15	24.93	ND	8.12	ND
2-nonanone	ND	ND	ND	ND	105.55
2-undecanone	ND	66.82	55.82	13.45	6.27
Total ketones	**328.00**	**321.57**	**355.27**	**488.22**	**514.04**
Alcohols					
Benzenemethanol	ND	290.15	234.33	170.04	192.22
2-butyl-ethanol	ND	109.23	202.12	66.99	107.82
2-methyl, 1- propanol	56.77	166.61	25.61	45.51	39.26
3-methyl-butanol	ND	ND	6.27	82.51	59.06
1-butanol	44.32	ND	53.53	46.55	8.14
3-methyl-2-butanol	99.35	68.61	43.36	51.02	66.63
1-pentanol	6.62	ND	ND	ND	ND
1-penten-3-ol	71.92	204.44	162.19	210.19	233.92
5-methoxy-1-pentanol	ND	102.42	92.92	88.52	88.09
1-hexanol	ND	ND	11.55	66.78	45.02
2-ethyl, 1-hexanol	43.33	6.01	ND	4.44	11.27
5-methyl-cyclohexanol	ND	ND	7.02	ND	6.25

ND: Non-detectable

Kapi-C: *Kapi* without inoculum, *Kapi*-B2, *Kapi*-B4 and *Kapi*-B6: *Kapi* inoculated with *Bacillus* spp. K-C3 at the level of 10^2, 10^4 and 10^6 CFU/ g dry weight sample, respectively.

Table 38. Volatile compounds of *Kapi* inoculated without and with *Bacillus* spp. K-C3 at different levels (Cont.)

Volatile compounds	Peak area (Abundance)×10^6				
	Fresh shrimp	*Kapi*-C	*Kapi*-B2	*Kapi*-B4	*Kapi*-B6
1,5-octadiene-3-ol	ND	ND	9.15	49.18	72.89
2,7-octadiene-1-ol	22.66	ND	2.03	ND	ND
1-octen-3-ol	204.25	ND	ND	47.02	40.05
1-dodecanol	ND	ND	ND	11.34	20.25
Total alcohols	**549.22**	**947.47**	**850.08**	**940.05**	**990.87**
***S*-containing compounds**					
Dimethyl-disulfide	275.01	ND	ND	ND	ND
Dimethyl-trisulfide	64.57	ND	ND	ND	ND
Methanethiol	36.63	88.86	85.22	68.92	96.10
Total *S*-containing compounds	**376.21**	**88.86**	**85.22**	**68.92**	**96.10**
***N*-containing compounds**					
N,N-dimethyl-methylamine	ND	ND	16.85	6.17	6.23
3-ethyl-4-methyl-pyridine	ND	10.00	22.36	67.57	44.02
1-pentyl-1-pyrrole	ND	ND	ND	3.99	10.15
Methyl-pyrazine	52.06	177.89	102.86	215.43	202.03
2-ehtyl-6-methyl-pyrazine	155.72	314.51	306.17	333.22	304.14
3-ethyl-5-methyl-pyrazine	203.38	219.88	108.95	204.37	148.76
2,3-diethyl, 5-methyl-pyrazine	52.31	165.22	167.83	152.49	206.99
2,5-dimethyl-pyrazine	156.89	203.41	304.05	299.18	299.55
2,6-dimethyl-pyrazine	55.44	55.16	105.52	76.18	88.98
3-ethyl-2,5-dimethyl-pyrazine	602.27	609.22	662.22	595.91	604.15
2-ethyl-3,5-dimethyl-pyrazine	103.33	222.55	167.23	222.41	206.87
2,6-diethyl-3,5-dimethylpyrazine	ND	ND	14.15	44.66	103.76
Trimethyl-pyrazine	ND	13.36	60.04	45.20	30.09
2,3,5-trimethyl-6-ethyl-pyrazine	ND	ND	ND	11.04	10.19
Total *N*-containing compounds	**1381.40**	**1991.20**	**2038.23**	**2277.82**	**2265.91**
Hydrocarbons					
3-dodecyne	ND	ND	11.02	22.13	9.88
3-tetradocene	4.32	49.88	22.99	19.86	33.04
2,3-butanediene	ND	16.12	ND	ND	ND
2-undecane	16.25	9.13	22.24	30.45	29.18
Hexadecane	24.99	ND	ND	ND	ND
2,6-cyclohexadiene	33.06	ND	ND	ND	ND
3-ethyl-hexa-1,5-diene	ND	44.23	60.06	55.49	56.27
Octane	ND	ND	22.03	10.99	9.05
2-octene	ND	ND	11.26	30.95	33.42
Total hydrocarbons	**78.62**	**119.36**	**149.60**	**169.87**	**170.84**

ND: Non-detectable

Kapi-C: *Kapi* without inoculum, *Kapi*-B2, *Kapi*-B4 and *Kapi*-B6: *Kapi* inoculated with *Bacillus* spp. K-C3 at the level of 10^2, 10^4 and 10^6 CFU/ g dry weight sample, respectively.

Table 38. Volatile compounds of *Kapi* inoculated without and with *Bacillus* spp. K-C3 at different levels (Cont.)

Volatile compounds	Peak area (Abundance)×10^6				
	Fresh shrimp	*Kapi*-C	*Kapi*-B2	*Kapi*-B4	*Kapi*-B6
Others					
Propanoic acid	ND	22.95	ND	ND	ND
Butanoic acid	ND	9.14	40.22	65.31	56.56
2-methyl-butanoic acid	ND	ND	11.23	22.55	22.09
Octanoic acid	ND	ND	8.09	ND	ND
Phenol	198.58	205.11	102.44	99.65	155.22
1H-Indole	77.63	292.96	398.53	355.66	367.02
Total others	**276.21**	**530.16**	**560.51**	**543.17**	**600.89**
Total Intensity	**3132.09**	**4361.42**	**4870.23**	**5497.08**	**5586.23**

ND: Non-detectable
Kapi-C: *Kapi* without inoculum, *Kapi*-B2, *Kapi*-B4 and *Kapi*-B6: *Kapi* inoculated with *Bacillus* spp. K-C3 at the level of 10^2, 10^4 and 10^6 CFU/ g dry weight sample, respectively.

Generally, aldehydes and ketones were more likely generated from lipid oxidation during fermentation (Michihata *et al.,* 2002). The higher abundance of aldehydes and ketones were attained in the inoculated *Kapi* at all levels, compared with *Kapi-C.* This was in accordance with the higher lipolysis and lipid oxidation when inoculated with starter culture as indicated by the higher FFA content and lipid oxidation products, respectively (Figure 37). Five aldehydes were found in fresh shrimp. Those included 2-methyl-butanal, 3-methyl-butanal, 2-hexanal, 2-octenal and benzaldehyde. Those aldehydes were also detected in *Kapi*, except 2-octenal, which was not present in *Kapi-B4* and *Kapi-B6.* Some aldehydes including 2-ethyl-butanal, pentanal and heptanal were generated during *Kapi* production and found only in the final product. However, 2-ethyl-butanal was not detected in *Kapi-C.* 2- and 3-methyl-butanal as well as benzaldehyde were the most abundant aldehydes found in shrimp pastes (Cha and cadwallader, 1995; Pongsetkul *et al.,* 2017a). Varlet and Fernandez (2010) reported that the Strecker aldehydes including 2-methyl-butanal and 3-methyl-butanal were generated during fermentation by microorganisms via transamination and decarboxylation of free amino acids. Those compounds exhibited cheese/feet, sour/fermented and butter flavors in many fermented fishery products.

Thirteen ketones were identified. Each samples had varying types and abundance of ketones. 1-phenyl-ethanone, 1-(2-aminophenyl)-ethanone, 2-petanone, 2-

hexanone and 2-undecanone were detected in all *Kapi* samples. Similar to aldehydes, many ketones were generated during processing of *Kapi* since they were not found in fresh shrimp. Moreover, the abundance of ketones was increased as the level of inoculation increased. Generally, abundance of 2-hexanone was drastically increased with increasing inoculation levels, indicating the ability of *Bacillus* spp. K-C3 for generating this compound in *Kapi.* Kim *et al.* (1996) reported that *Bacillus* species SSA3-2M1 was responsible for the production of 2-hexanone, which was related to the formation of sour, blue-cheese and pungent off-odor in traditional Korean soy sauce. Vegetarian soybean *kapi* inoculated with *B. subtilis* IS4 had the higher amount of some ketones including 2-octanone, 2-hexanone and 2-nonanone, compared with commercial *Kapi* as reported by Wittanalai *et al.* (2011). Similarly, 2-octanone was found only in *Kapi-B4* and *Kapi-B6,* while 2-nonanoe was detected only in *Kapi-B6* sample. Ketones were generated by microbial enzymatic actions on lipids and/or amino acids, or by the Maillard reaction during shrimp paste fermentation and seemed to be responsible for the desirable flavor, particularly cheesy note (Cha and Cadwallader, 1995). However, their aroma contributions might be minimal because of high odor threshold values.

Various alcohols were also detected. 1-octen-3-ol was the most dominant alcohol found in fresh shrimp. 1-octen-3-ol is rapidly formed from oxidation of arachidonic acid by lipoxygenase and play a role in off-flavor in many fresh seafood due to its low odor threshold (Varlet and Fernandez, 2010). However, this compound disappeared in *Kapi-C* and *Kapi-B2*, while much lower abundance was obtained in *Kapi-B4* and *Kapi-B6.* Some alcohols were found in all *Kapi* samples, but not noticeable in fresh shrimp. Those included benzenemethanol, 2-butyl-ethanol and 5-methoxy-1-pentanol. Compared to fresh shrimp, all *Kapi* samples had the higher abundance of alcohols. However, some alcohols including 3-methyl-butanol, 1-hexanol, 1,5-octadiene-3-ol and 1-dodecanol were found only in the inoculated *Kapi* samples. The result suggested that alcohols were also generated during *Kapi* production and the formation of some alcohols was induced by the starter culture added. This could be responsible for the unique odor or flavor of the inoculated *Kapi.* However, Cha and Cadwallader (1995) suggested that alcohols could not have the marked impact on *Kapi* flavor due to their high flavor thresholds.

It was noted that *S*-containing compounds obtained in fresh shrimp were higher than those found in all *Kapi* samples. In fresh shrimp, three *S*-containing compounds including dimethyl-disulfide, dimethyl-trisulfide and methanethiol were identified, while only methanethiol was still obtained in the final product. Normally, volatile sulfur compounds in fermented food were from the metabolism of methionine (Landaud *et al.,* 2008). Methionine is the one of dominant amino acids found in shrimp *A. vulgaris*, however this amino acid was decreased throughout *Kapi* production (Pongsetkul *et al.,* 2017b). This was in agreement with the higher methanethiol of the final product, compared with fresh shrimp. Varlet and Fernandez (2010) also reported that *S*-containing compounds were produced by enzymatically degradation of amino acid, particularly methionine and cysteine. Therefore, the low abundance of *S*-containing compound in *Kapi* might be due to the loss in activities of *S*-containing compound producing-enzymes, both endogenous and microbial enzymes, during *Kapi* production. Also, these compounds might be decomposed into other compounds. Furthermore, it was presumed that starter culture might not contain *S*-containing compound producing-enzymes. Owing to their low abundance in *Kapi*, *S*-containing compounds might not have a paramount impact on *Kapi* flavor or odor.

N-containing compounds were the major volatiles found in fresh shrimp and all *Kapi* samples. Among all *N*-containing compounds, 3-ethyl-2,5-dimethyl-pyrazine had the highest abundance in all samples. Eight *N*-containing compounds were obtained in fresh shrimp and were still found in all *Kapi* samples. Those included methyl-pyrazine, 3-ethyl-5-methyl-pyrazine, 2,5-dimethyl-pyrazine, etc. 3-ethyl-4-methyl-pyridine and trimethyl-pyrazine were not found in fresh sample, but were detected in all *Kapi* samples. It was noted that some *N*-containing compounds were found only in the inoculated *Kapi.* Those consisted of *N,N*-dimethyl-methylamine, 1-pentyl-1-pyrrole, 2,6-diethyl-3,5-dimethylpyrazine and 2,3,5-trimethyl-6-ethyl-pyrazine. Most *N*-containing compounds in *Kapi* were pyrazine derivatives. The formation of pyrazines is associated with heating and metabolic activities of microorganisms (Cha *et al.,* 1983). Pyrazines and heterocyclic nitrogen compounds could also be associated with MRPs, and contribute to preferable odor in many processed food (Cha *et al.,* 1983). Therefore, MRPs formed during *Kapi* production, not only affected brown color of the product, but also determined flavor or odor of *Kapi*

to some extent. Higher formation of MRPs was observed when *Kapi* was inoculated by *Bacillus* spp. K-C3 (Table 37). This was related with the higher abundance of pyrazine derivatives found in the inoculated samples. Pyrazines were the major volatile compounds present in various fermented foods and seemed to be the most potent contributors to their characteristic odor including *Sikhae* (Korean fermented sardine) (Cha *et al.,* 1983), *Ishiru* (Japanese fish sauce) (Michihata *et al.,* 2002), *Thua-nao* (vegetarian soybean kapi) (Wittanalai *et al.,* 2011), fermented dried shrimp (Acetes chinensis) (Lu *et al.,* 2011) as well as *Kapi* (Cha and Cadwallader, 1995; Pongsetkul *et al.,* 2015), etc.

Hydrocarbons were obtained at the low abundance. Only 3-tetradocene and 2-undecane were found in fresh shrimp and all *Kapi* samples. Hexadecane and 2,6-cyclohexadiene were detected in fresh shrimp but disappeared after *Kapi* processing, while 3-ethyl-hexa-1,5-diene was only found in all *Kapi* products. Moreover, some hydrocarbons including 3-dodecyne, octane and 2-octane were noticeable only in the inoculated samples. Mainly, hydrocarbons are derived from lipids by thermal homolysis or autoxidation of long-chain fatty acids (Lu *et al.,* 2011). Cha and Cadwallader (1995) reported that alkanes and alkenes are mainly formed from auto-oxidation of fatty acids released from triglycerides. However, hydrocarbons might not affect the final flavor or odor because of very low abundance.

For other compounds, phenol and indole were obtained in fresh shrimp. These compounds were still remained in all *Kapi* samples. Normally, phenol was reported to give an undesirable aroma in seafood (Cha and Cadwallader, 1995). Indole is the degradation product from tryptophan (Michihata *et al.,* 2002). Indole, even at low concentrations, has a profound role in marine-like, salty, shrimpy, and iodine-like off flavors in shrimp (Lu *et al.,* 2011). Volatile acids were also identified in *Kapi*, but were not obtained in fresh shrimp. Butanoic acid was found in all *Kapi* samples, whereas 2-methyl-butanoic acid was noted only in the inoculated *Kapi* samples. Giri *et al.* (2010) revealed that volatile acids, such as 2-methyl-butanoic acid and butanoic acid, were produced as a result of either lipolysis or from amino acid metabolism in terms of valine deamination. Most volatile acids were found in fish sauce and responsible for cheesy notes, which were desirable characteristics of the product (Michihata *et al.,* 2002).

Overall, volatiles of all *Kapi* samples (final products) had the higher abundance, compared to those found in fresh shrimp, indicating that some biochemical changes occurred during *Kapi* production, especially protein and lipid degradation/ decompositions. Degradation of proteins, lipolysis, lipid oxidation, Maillard reaction, interaction between MRPs with lipid-oxidized products and other reactions more likely contributed to the formation of volatile compounds, which were associated with its final flavor and odor characteristics. Among all *Kapi* samples, *Kapi-B4* had the highest abundance of volatiles, followed by *Kapi-B6* and *Kapi-B2,* respectively. Inoculation with starter culture therefore led to the increase in abundance of volatiles generated during fermentation of *Kapi.*

13.6 Conclusion

The inoculation of *Bacillus* spp. K-C3, isolated from commercial *Kapi*, at the level of 10^2-10^6 CFU/g dry weight sample, resulted in the higher protein and lipid degradation/decomposition. This favored the development of *Kapi* characteristics including the color development, especially brown color, lipid oxidations, antioxidative activities as well as volatile compounds. Fermentation time could be shortened, compared with the traditional fermentation process of this product. Therefore, *Bacillus* spp. K-C3, especially at a level of 10^4 CFU/g dry weight sample, could be considered as the potential starter culture used for *Kapi* production.

13.7 References

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CHAPTER 14

CONCLUSION AND SUGGESTION

14.1 Conclusions

1. Different commercial *Kapi,* obtained from various places of Thailand, had varying compositions and characteristics e.g. color, amino acids composition, volatile compounds, etc. However, all *Kapi* samples were rich in proteins and also possessed antioxidant activities.

2. Both shrimp *A. vulgaris* and *M. lanchesteri* contained serine proteases (trypsin and chymotrypsin) as major proteases with optimum pH and temperature of 7, 60°C and 8, 60°C, respectively. Those proteases existed throughout *Kapi* production.

3. Post-mortem storage time of shrimp prior to salting affected the final characteristics and quality of *Kapi.* The delay in salting of shrimp *A. vulgaris* and *M. lanchesteri* should not exceed 6 and 12 h, respectively, in which the resulting *Kapi* still had the sensorial property equivalent to that prepared from fresh shrimp.

4. Shrimp *A. vulgaris* have the higher potential to be used as alternative raw material for *Kapi* production than shrimp *M. lanchesteri* as indicated by the higher fermentation rate (proteolysis and lipolysis). Typical characteristics could be developed at a faster rate. The former also had the higher antioxidative activities as well as sensory score than the latter.

5. Properties of *Kapi*, produced from shrimp *A. vulgaris*, were governed by all processes including salting, drying and fermentation. Salting and drying processes were mainly implemented to lower moisture content and A_w. Protein/lipid degradation as well as the development of color and volatiles significantly occurred during fermentation period.

6. The major lipid constituents of fresh shrimp *A. vulgaris* and *Kapi* were TAG and FFA, respectively. *Kapi* was rich in PUFA, especially C20:5(n-3) (EPA), which were accumulated in PE and PC. These PUFAs still remained in *Kapi*. Lipid oxidation occurring throughout *Kapi* production affected lipid constituents. These changes could be associated with final characteristics of *Kapi*, especially odor and flavor.

7. The optimal condition for growth and production of extracellular enzymes including protease, lipase and chitinase of *Bacillus* spp. K-C3, isolated from commercial *Kapi*, was 25-35°C and pH 6-8 in the presence of 10% NaCl. Inoculation of this strain at the level of 10^4 CFU/g dry weight sample could enhance the development of *Kapi* via increasing proteolysis, lipolysis and formation of volatile compounds, which were related with the desired quality.

14.2 Suggestions

1. Effect of packaging as well as storage condition on quality and shelf-life of *Kapi* should be further studied.

2. Changes in some components, e.g. carotenoid, etc., and their interaction with other constituents, related with the changes in color of shrimp during *Kapi* production or storage should be examined.

3. Fermentation condition or microbial starter should be optimized to improve the desirable flavor of *Kapi* and shorten the fermentation period.

APPENDIX

Alternative shrimp for *Kapi* production

Acetes vulgaris

Body length 15.6±1.4 mm
Wet weight 0.0413±0.0098 g
(n=20)

Macrobrachium lanchesteri

Body length 27.5±1.9 mm
Wet weight 0.0701±0.0107 g
(n=20)

Fermentation process of *Kapi*

Compacted pound dried salted shrimp into glass jar into container

Wrap with aluminium foil

Cover with plastic bag

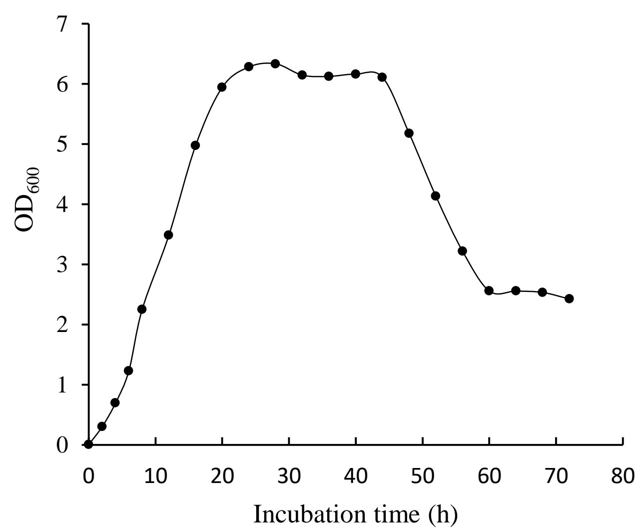
Cover with cheesecloth

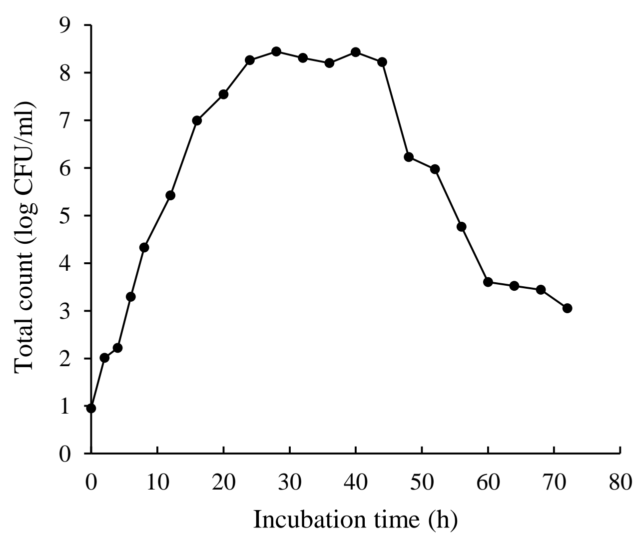
***Kapi* produced from alternative shrimp**

Kapi from *Acetes vulgaris*

Kapi from *Macrobrachium lanchesteri*

Growth curve of *Bacillus* spp. K-C3





List of 16S rRNA gene sequencing of bacterial

Specie: *Bacillus subtilis*

Strain: K-C3

Base pair: 1348

Similarity: 99.01% similar to *B. subtilis* subsp. subtilis strain 168

Gene sequence:

CGCCAATGGCTGCATACTATACATGCAAGTCGAGCGGACAGATGGGAGCTTGCTC
CCTGATGTTAGCGGCGGACGGGTGAGTAACACGTGGGTAACCTGCCTGTAAGACT
GGGATAACTCCGGGAAACCGGGGCTAATACCGGATGGTTGTTTGAACCGCATGG
TTCAGACATAAAAGGTGGCTTCGGCTACCACTTACAGATGGACCCGCGGCGCATT
AGCTAGTTGGTGAGGTAACGGCTCACCAAGGCGACGATGCGTAGCCGACCTGAG
AGGGTGATCGGCCACACTGGGACTGAGACACGGCCCAGACTCCTACGGGAGGCA
GCAGTAGGGAATCTTCCGCAATGGACGAAAGTCTGACGGAGCAACGCCGCGTGA
GTGATGAAGGTTTTCGGATCGTAAAGCTCTGTTGTTAGGGAAGAACAAGTGCCGT
TCAAATAGGGCGGCACCTTGACGGTACCTAACCAGAAAGCCACGGCTAACTACG
TGCCAGCAGCCGCGGTAATACGTAGGTGGCAAGCGTTGTCCGGAATTATTGGGCG
TAAAGGGCTCGCAGGCGGTTTCTTAAGTCTGATGTGAAAGCCCCCGGCTCAACCG
GGGAGGGTCATTGGAAACTGGGGAACTTGAGTGCAGAAGAGGAGAGTGGAATTC
CACGTGTAGCGGTGAAATGCGTAGAGATGTGGAGGAACACCAGTGGCGAAGGCG
ACTCTCTGGTCTGTAACTGACGCTGAGGAGCGAAAGCGTGGGGAGCGAACAGGA
TTAGATACCCTGGTAGTCCACGCCGTAAACGATGAGTGCTAAGTGTTAGGGGGTT
TCCGCCCCTTAGTGCTGCAGCTAACGCATTAAGCACTCCGCCTGGGGAGTACGGT
CGCAAGACTGAAACTCAAAGGAATTGACGGGGGCCCGCACAAGCGGTGGAGCAT
GTGGTTTAATTCGAAGCAACGCGAAGAACCTTACCAGGTCTTGACATCCTCTGAC
AATCCTAGAAGATAGGACGTCCCCTTCGGGGGCAGAGTGACAGGTGGTGCATGG
TTGTCGTCAGCTCGTGTCGTGAGATGTTTGGGTTAAGTCCCGCAACGAGCGCAAC
CCTTGATCTTAGTTGCCAGCATTCAGTTGGGCACTCTAAGGTGACTGCCGGTGAC
AAACCGGAAGAAAAGGGGGGGATGAAGTTCAAATCATCCAGGCCCCTTAAGAAC
CGGGGCTAACACCCTGGCTACAATGGGACAGAACAAAGGGGACCCAAAACCCCC
GAGGTTAAACCCATCCCCCAAAATTTGTTTTCCAATTTCGGATACCCAATCTGGC
AACTCCAACGGGCGGGAAACTGGGAAACCCCCTTAATACC

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