Protein Hydrolysate from Tuna Roes: Characteristics and Use as Antioxidative Emulsifier in Emulsion Food Systems

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A Thesis Submitted in Fulfillment of the Requirements for the Degree of
Doctor of Philosophy in Food Science and Technology
Prince of Songkla University
2014

Thesis Title	Protein Hydrolysate from Tuna Roes: Characteristics and Use as Antioxidative Emulsifier in Emulsion Food Systems
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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 โปรตีนไฮโดรไลเสตจากไข่ปลาทูน่า: คุณลักษณะ และการใช้เป็นอิมัลซิ-ไฟเออร์ที่มีฤทธิ์ต้านอนุมูลอิสระในระบบอาหารอิมัลชัน

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บทคัดย่อ

จากการศึกษาองค์ประกอบทางเคมีไข่ปลาทูน่า 3 สายพันธุ์ ได้แก่ ปลาโอแถบ ปลาโอดำ และปลาโอลาย พบว่า ไข่ปลาประกอบด้วย ความชื้นร้อยละ 72.17-73.03 โปรตีนร้อยละ 18.16-20.15 ไขมันร้อยละ 3.29-5.68 และเถ้าร้อยละ 1.79-2.10 โดยไข่ปลาทั้งสามสายพันธุ์ที่ไม่ผ่านการกำจัดไขมันและที่ผ่านการกำจัดไขมัน มีโปรตีนที่มีน้ำหนักโมเลกุล 97 กิโลดาลตัน เป็นองค์ประกอบหลัก และมีกรดกลูตามิกและกลูตามีนเป็นกรดอะมิโนที่พบมากที่สุด (12.18-12.65 กรัม/100 กรัมโปรตีน) ขณะที่โปแตสเซียม โซเดียม และฟอสฟอรัส เป็นธาตุหลักในไข่ปลาที่ผ่านการกำจัดไขมัน ลิปิดจากไข่ปลาทูน่ามีฟอสโฟลิปิดเป็นองค์ประกอบหลัก (ร้อยละ 51.22-54.90 ของลิปิดทั้งหมด) และมีปริมาณ docosahexaenoic acid สูง (DHA, ร้อยละ 20.53-26.19 ของลิปิดทั้งหมด) และมีปริมาณ eicosapentaenoic acid (EPA) ร้อยละ3.8-4.62 ของลิปิดทั้งหมด ทั้งนี้ลิปิดจากไข่ปลาทูน่ามีปริมาณคอเลสเตอรอลอยู่ในช่วง 94–172 มิลลิกรัม/100 กรัมลิปิด

เมื่อนำไข่ปลาโอแถบที่ผ่านการกำจัดไขมันและมีปริมาณโปรตีนสูงสุด (ร้อยละ 20.15) มาย่อยด้วย Alcalase 2.4 L ได้เป็นโปรตีนไฮโดรไลเสตจากไข่ปลาโอแถบ (SRPH) ซึ่งเมื่อนำ SRPH ที่ระดับการย่อย (DH) ต่างๆ และที่ความเข้มข้นแตกต่างกัน ไปทดสอบกิจกรรมออกฤทธิ์ต้านอนุมูลอิสระ และสมบัติเชิงหน้าที่ พบว่า ระดับ DH เพิ่มขึ้น กิจกรรมออกฤทธิ์จับอนุมูลอิสระ DPPH และ ABTS ตลอดจนความสามารถในการรีดิวซ์ลดลง ขณะที่ความสามารถในการจับโลหะและกิจกรรมออกฤทธิ์จับซุปเปอร์ออกไซด์อิออนเพิ่มขึ้น ($p < 0.05$) อย่างไรก็ตาม ความสามารถในการจับโลหะลดลง เมื่อ SRPH มี DH สูงกว่า 30 ทั้งนี้พบว่า ความสามารถในการออกฤทธิ์ทุกกิจกรรม ยกเว้นกิจกรรมออกฤทธิ์จับซุปเปอร์ออกไซด์ไอออน เพิ่มขึ้นตามความเข้มข้นของ SRPH ($p < 0.05$) นอกจากนี้การย่อยด้วย Alcalase ยังสามารถเพิ่มสมบัติการละลายโปรตีนได้มากกว่า ร้อยละ 80 ในช่วงพีเอชที่กว้าง (2-10) ความเข้มข้นของ SRPH มีผลต่อสมบัติระหว่างพื้นผิวโดยขึ้นกับ DH สำหรับค่าดัชนีความสามารถในการเกิดอิมัลชัน (EAI) และความคงตัวของฟอง (FS) มีค่าสูงสุดสำหรับ SRPH ที่มี DH ต่ำ (ร้อยละ 5) ซึ่ง SRPH ที่ระดับ DH ร้อยละ 5 ประกอบด้วยสองพีคหลักซึ่ง

มีน้ำหนักโมเลกุลคือ 57.8 และ 5.5 กิโลดาลตันโดยแฟรกชันที่มีน้ำหนักโมเลกุล 5.5 กิโลดาลตัน มีความสามารถในการจับโลหะ และมีกิจกรรมออกฤทธิ์จับอนุมูลอิสระ ABTS สูงสุด

การแยกและจำแนกคุณสมบัติของเปปไทด์ที่ออกฤทธิ์ทางชีวภาพจาก SRPH ที่ระดับ DH ร้อยละ 5 โดยการทำบริสุทธิ์เป็นลำดับ (อัลตราฟิลเทรชัน คอลัมน์โครมาโทรกราฟีแบบแลกเปลี่ยนประจุบวก และคอลัมน์*โครมาโทกราฟี*ของเหลวสมรรถนะสูงแบบวัฏภาคย้อนกลับ) พบว่า มีสองแอคทีฟแฟรกชัน จากสองแฟรกชันดังกล่าวพบเปปไทด์ที่มีความสำคัญ 7 เปปไทด์ ซึ่งประกอบไปด้วยกรดอะมิโน 6-11 ตัว และเมื่อจำแนกลำดับกรดอะมิโนของเปปไทด์โดย LC-MS/MS พบว่าเปปไทด์มีลำดับกรดอะมิโนดังนี้ DWMKGQ MLVFAV MCYPAST FVSACSVAG LADGVAAPA YVNDAATLLPR และ DLDLRKDLYAN เมื่อสังเคราะห์เปปไทด์ดังกล่าว แล้วนำไปทดสอบสมบัติในการยับยั้งกิจกรรม Angiotensin I converting enzyme (ACE) และการออกฤทธิ์ต้านอนุมูลอิสระ ปรากฏว่า เปปไทด์ MLVFAV ซึ่งมีความสามารถในการยับยั้งกิจกรรม ACE สูงสุด และไม่มีกิจกรรมออกฤทธิ์ต้านอนุมูลอิสระ ขณะที่เปปไทด์ DLDLRKDLYAN มีกิจกรรมความสามารถออกฤทธิ์จับอนุมูลอิสระ ABTS และจับ singlet oxygen

จากการศึกษาผลการเติม SRPH ที่มีระดับ DH ร้อยละ 5 ที่ระดับความเข้มข้นต่างๆ (ร้อยละ 0-3) ในไส้กรอกอิมัลชันที่เตรียมจากเนื้อปลาดุกอุยเสริมกรดไขมันโอเมก้า 3 จากไข่ปลาโอแถบ ต่อคุณสมบัติและความคงตัวต่อการเกิดออกซิเดชัน พบว่า การเติม SRPH มีผลให้ hardness cohesiveness และ resilience ของไส้กรอกมีค่าเพิ่มขึ้น ($p < 0.05$) และเม็ดไขมันในไส้กรอกมีความละเอียดมากขึ้น เมื่อเติม SRPH ที่ระดับความเข้มข้นสูงขึ้น นอกจากนี้การเติม SRPH สามารถชะลอการเกิดออกซิเดชันของไส้กรอกระหว่างการเก็บรักษา 12 วัน สังเกตจากค่า peroxide value (PV) และ thiobarbituric acid reactive substances (TBARS) ที่ต่ำกว่า เมื่อเปรียบเทียบกับชุดควบคุมที่ไม่เติม SRPH ไส้กรอกที่เติม SRPH ร้อยละ 3 ยังมีปริมาณ DHA และ EPA คงเหลือมากกว่าร้อยละ 80 ภายหลังการเก็บรักษา 12 วัน โดยที่การเติม SRPH ไม่มีผลต่อคุณภาพทางประสาทสัมผัส และสามารถป้องกันการเกิดกลิ่นหืนได้ แต่อย่างไรก็ตาม SRPH ไม่สามารถยับยั้งการเจริญเติบโตของเชื้อจุลินทรีย์ได้

เมื่อศึกษาการใช้ SRPH ที่ระดับ DH ร้อยละ 5 เป็นอิมัลซิไฟเออร์ในอิมัลชันประเภทน้ำมันในน้ำ โดยใช้แรงดันในการโฮโมจิไนซ์ที่ระดับต่างๆ (2,000, 3,000 และ 4,000 ปอนด์ต่อตารางนิ้ว) ต่อความคงตัวของอิมัลชัน พบว่า ในระหว่างการเก็บรักษาอิมัลชันนาน 14 วัน ขนาดอนุภาค ดัชนีการเกิดครีม flocculation factor (F_f) ตลอดจน coalescence index (C_i) ในอิมัลชันที่มี SRPH เป็นองค์ประกอบมีค่าเพิ่มขึ้น โดยเฉพาะอิมัลชันที่เตรียมที่ระดับแรงดันโฮโมจิไนซ์

3,000 และ 4,000 ปอนด์ต่อตารางนิ้ว ($p < 0.05$) อย่างไรก็ตาม แรงดันโฮโมจิไนซ์ที่ระดับ 2,000 ปอนด์ต่อตารางนิ้ว มีความเหมาะสมต่อการเตรียมอิมัลชันที่มี SRPH เป็นองค์ประกอบ การเติมกรดแทนนิก (TA) ที่ผ่านการออกซิเดชัน (OTA) ร้อยละ 1 (น้ำหนักต่อปริมาตร) ก่อนและหลังการทำให้เกิดอิมัลชัน ในอิมัลชันที่มี SRPH ระดับความเข้มข้นต่างๆ (ร้อยละ 5 และ 10 น้ำหนักต่อปริมาตร) ให้อิมัลชันที่มีความคงตัวแตกต่างกัน โดยอิมัลชันที่ให้ความคงตัวด้วย SRPH ที่ระดับความเข้มข้นร้อยละ 10 มีค่า F_f และ C_i ลดลง เมื่อเติม OTA ภายหลังการทำให้เกิดอิมัลชัน ($p < 0.05$) และเมื่อพิจารณาความคงตัวของอิมัลชันที่เติม OTA ภายหลังการทำให้เกิดอิมัลชัน ที่ระดับความเข้มข้นของ OTA แตกต่างกัน (ร้อยละ 0-2 โดยน้ำหนัก) พบว่า ความเข้มข้นของ OTA เพิ่มขึ้นมีผลทำให้ขนาดอนุภาค (d_{43} และ d_{32}) ของอิมัลชันตลอดจน C_i และ F_f มีค่าลดลง ขณะที่ค่าศักย์ซีตาสูงขึ้น โดย OTA มีผลเหนี่ยวนำการเชื่อมประสานระหว่างเปปไทด์เล็กน้อย โดยเฉพาะ เปปไทด์ที่อยู่บริเวณระหว่างผิวน้ำและน้ำมัน ทั้งนี้อิมัลชันที่ประกอบด้วย SRPH และเติม OTA สามารถชะลอการเกิด TBARS ได้โดยแปรผันตามความเข้มข้นของ OTA ($p < 0.05$)

ไมโครเอนแคปซูเลชันน้ำมันปลาที่ใช้ SRPH ที่ระดับ DH ร้อยละ 5 เป็นสารห่อหุ้มทั้งในสภาวะที่ไม่มีและมี TA หรือ OTA สามารถทำได้โดยการทำแห้งแบบพ่นฝอยไมโครอิมัลชัน โดยทั่วไป เมื่อใช้ SRPH เป็นสารห่อหุ้ม ไมโครแคปซูลที่ได้มักมี*ความสามารถในการ*ห่อหุ้ม (encapsulation efficiency, EE) ต่ำ การเติม TA หรือ OTA ร่วมกับ SRPH ทำให้ได้ผลิตผลน้ำมันปลาซึ่งผ่านการห่อหุ้มมีลักษณะเป็นทรงกลม มีค่า EE สูง และมีขนาดอนุภาคเล็กกว่าที่ได้จากการห่อหุ้มด้วย SRPH เพียงอย่างเดียว ($p < 0.05$) นอกจากนี้การเติม TA ยังสามารถชะลอการเกิดออกซิเดชันของลิปิดในไมโครแคปซูลได้ ดังจะเห็นได้จากค่า PV และ TBARS ที่ต่ำ ตลอดการเก็บรักษา 4 สัปดาห์ เมื่อเปรียบเทียบกับชุดการทดลองที่ห่อหุ้มด้วย SRPH เพียงอย่างเดียว ทั้งนี้พบว่าชุดการทดลองที่มี OTA ร่วมกับ SRPH ให้ไมโครแคปซูลที่มีค่า EE สูงกว่าในชุดการทดลองที่มี TA ร่วมกับ SRPH ดังนั้น SRPH ที่ระดับ DH ร้อยละ 5 จึงสามารถใช้เป็นอิมัลซิไฟเออร์และสารห่อหุ้มอีกทางเลือกหนึ่ง เมื่อเตรียมอิมัลชันโดยการเติม TA หรือ OTA ร่วมกับการใช้แรงดันในการโฮโมจิไนซ์ที่เหมาะสมก่อนการทำแห้งแบบพ่นฝอย

Thesis Title Protein Hydrolysate from Tuna Roes: Characteristics and Use as Antioxidative Emulsifier in Emulsion Food Systems

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Major Program Food Science and Technology

Academic Year 2013

ABSTRACT

The chemical compositions of roes from three tuna species including skipjack (*Katsuwonous pelamis*) (SJK), tongol (*Thunnus tonggol*) (TGL) and bonito (*Euthynnus affinis*) (BNO) were investigated. Tuna roes contained 72.17-73.03% moisture, 18.16-20.15% protein, 3.29-5.68% lipid and 1.79-2.10% ash. Non-defatted and defatted roes from all species had the protein with a molecular weight (MW) of 97 kDa as the major component. Glutamic acid and glutamine were the most abundant amino acids (12.18-12.65 g/100g protein), whereas K, Na and P were the major elements in defatted tuna roes. Crude lipids from tuna roes contained phospholipids as the major component (51.22-54.90% of total lipids) with high content of docosahexaenoic acid (DHA; 20.53-26.19% of total lipids) and eicosapentaenoic acid (EPA, 3.80-4.62% of total lipid). Cholesterol contents were in the range of 94–172 mg/100 g lipids.

The defatted SJK having the highest protein content (20.15%) was subjected to hydrolysis using Alcalase 2.4 L. Protein hydrolysate from skipjack roe (SRPH) with different degrees of hydrolysis (DH) at various concentrations was tested for antioxidative activites and functional properties. As DH increased, the reduction in DPPH, ABTS radicals scavenging activities and reducing power were noticeable, whereas the increased metal chelating and superoxide scavenging activities were attained ($p < 0.05$). However, DH of SRPH above 30% resulted in gradual decrease in chelating activity. All activities increased as the concentration of SRPH increased except superoxide anion radical scavenging activity ($p < 0.05$). Hydrolysis using Alcalase could increase the protein solubility to above 80% over a wide pH range (2-10). Concentrations of SRPH determined interfacial properties

differently, depending on DH. The highest emulsion ability index (EAI) and foam stability (FS) of SRPH were observed at low DH (5%) ($p < 0.05$). SRPH with 5% DH contained two major peaks with MW of 57.8 and 5.5 kDa. The strongest metal chelating and ABTS radical scavenging activities could be observed for fraction with MW of 5.5 kDa.

Bioactive peptides from SRPH with 5% DH were isolated and characterized. With consecutive purification steps (ultrafiltration, cation exchange column chromatography and reverse phase high performance liquid chromatography), two active fractions were obtained. Seven dominant peptides with 6-11 amino acid residues including DWMKGQ, MLVFAV, MCYPAST, FVSACSVAG, LADGVAAPA, YVNDAATLLPR and DLDLRKDLYAN were identified using LC-MS/MS. Peptides were then synthesized and analyzed for ACE-inhibitory and antioxidative activities. MLVFAV exhibited the highest ACE inhibitory activity with no antioxidative property, while DLDLRKDLYAN showed the highest metal chelating activity, ABTS radical and singlet oxygen scavenging activities.

The effect of incorporation of SRPH with 5% DH at various levels (0-3%) on the properties and oxidative stability of broadhead catfish (*Clarias macrocephalus*) emulsion sausage enriched with n-3 fatty acid from SJK roe was studied. The addition of SRPH increased hardness, cohesiveness and resilience of sausage ($p < 0.05$). Finer fat globules were visualized in the sample added with SRPH at higher levels. SRPH could retard lipid oxidation of sausage during 12 days of storage as indicated by the lower peroxide value (PV) and thiobarbituric acid reactive substances (TBARS), in comparison with the control. After 12 days, the sausage with 3% SRPH had the retained DHA and EPA, accounting more than 80%. Addition of SRPH had no effect on the organoleptic properties but could prevent the development of rancidity. Nevertheless, SRPH had no impact on inhibition of microbial growth.

When SRPH with 5% DH was used as an emulsifier in oil-in-water emulsion, homogenization pressure at various levels (2,000, 3,000 and 4,000 psi) had the impact on stability of emulsion containing SRPH. During 14 days of the extended

storage, particle size, creaming index, flocculation factor (*Ff*) and coalescence index (*Ci*) of SRPH containing emulsions sharply increased, especially in emulsions prepared at 3,000 and 4,000 psi ($p < 0.05$). However, homogenization pressure at 2,000 psi was appropriate for preparation of emulsion stabilized by SRPH. Addition of 1% (w/v) oxidized tannic acid (OTA) into emulsion containing SRPH at different levels (5 and 10%, w/v) before and after emulsification rendered different emulsion stability. Among all samples, emulsion stabilized by 10% SRPH showed the lower *Ci* and *Ff*, when 1% OTA was added after emulsification ($p < 0.05$). When OTA concentrations (0-2%, w/w) increased, particle size (d_{43} and d_{32}), C_i and F_f decreased but ζ-potential value increased. OTA slightly induced cross-linking of peptides, particularly those located at the interface. SRPH-emulsion containing OTA also inhibited the formation of TBARS in a dose-dependent manner ($p < 0.05$).

Microencapsulation of fish oil was achieved by spray drying micro-emulsion, in which SRPH with 5% DH was used as the wall material in the absence or presence of tannic acid (TA) or OTA. In general, microcapsule using SRPH had the lower encapsulation efficiency (EE). The incorporation of TA or OTA in combination with SRPH yielded spherical encapsulated fish oil with higher EE but lower particle size ($p < 0.05$), than SRPH alone. Moreover, the addition of TA could retard lipid oxidation of microcapsule as indicated by lower PV and TBARS value during 4 weeks of storage, compared with those using only SRPH. Nevertheless, OTA in conjunction with SRPH yielded the higher EE than TA. Therefore, SRPH with 5% DH could also be an alternative emulsifier and wall material for microencapsulation of fish oil when the addition of TA or OTA with appropriate homogenization pressure prior to spray drying.

ACKNOWLEDGEMENT

I would like to express my deepest appreciation and sincere gratitude to my advisor, Prof. Dr. Soottawat Benjakul of the Department of Food Technology, Faculty of Agro-Industry, Prince of Songkla University, for his kindness, guidance and assistance during my study. I also would like to thank for his invaluable suggestion and lessons for my academic knowledge and personal life. His determination and perseverance to train me to be a good researcher with responsibility, vigilance and honesty are always acknowledged.

I would like to express my profound gratitude to my co-advisor, Dr. Wonnop Visessanguan of National Center for Genetic Engineering and Biotechnology, (BIOTEC) for his kindness and helpful guidance in consultation. I also thank Asst. Prof. Dr. Jianping Wu of Department of Agricultural, Food & Nutritional Sciences, University of Alberta for his kindness, generous contribution and helpful guidance, particularly during my 9 months stay in Canada.

I am grateful to my examining committee, Asst. Prof. Dr. Manee Vittayanont of the Department of Food Technology and Assoc. Prof. Dr. Aran H-kittikun of the Department of Industrial Biotechnology, Faculty of Agro-Industry, Prince of Songkla University and Assoc. Prof. Dr. Jirawat Yongsawatdigul of the School of Food Technology, Institute of Agricultural Technology, Suranaree University of Technology for their kindness, comments and helpful suggestion.

My deep gratitude is also dedicated to my dear friends and colleagues of Fish Chemistry and Biochemistry lab (2205); Dr. Tanong Aewsiri, Dr. Chodsana Sriket, Dr. Sitthipong Nalinanon, Dr. Phanat Kittipattanabawon, Dr. Yaowapa Thiansilakul, Dr. Manat Chaijan, Dr. Sutheera Khantaphant, Ms. Suthasinee Yarnpakdee, Ms. Sirima Takeungwongtrakul, Mr. Theerapol Senphan, Mr. Sittichoke Sinthusamran, Mr.Samart Sai-Ut, Mr. Phakawat Tongnuanchan , Ms. Supatra Karnjanapratum, Ms. Sochaya Chanarat and Mr. Muralidharan Nagarajan. Thanks are also given to Thai and International students in Canada for their help; Ms. Chodchanok Vijarnsorn, Ms. Aramsri Meeprom, Ms. QiYi Li, Ms. Juan You and Ms.

Marina Offengenden, etc. for their most valuable help and friendship during my research at University of Alberta, Edmonton, Alberta, Canada. Finally, I would like to express my deepest appreciation to my beloved family for the great encouragement and support.

This study could not be succeeded without the financial support from the CHE Ph.D. Scholarships from the Office of the Higher Education Commission, Ministry of Education, Thailand and the Graduate School of Prince of Songkla Unversity. I gratefully acknowledge this financial support.

Rossawan Intarasirisawat

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CHAPTER 1
INTRODUCTION AND REVIEW OF LITERATURE

1.1 Introduction

Roe is referred to oocytes gathered in skeins (Mahmoud *et al.*, 2008). During oocyte maturation or vitellogenesis, the precursor protein is transferred into oocytes and converted to lipovitellin and phosvitin by specific proteolytic cleavage (Lubzens *et al.*, 2010). Lipovitellins and phosphoproteins, including phosvitins and phosvettes, are the major lipoprotein in fish egg yolk. Types and forms of yolk proteins may differ between different species of teleosts (Reading *et al.*, 2009). Fish roe contains 11% albumins, 75% ovoglobulin and 13% collagen (Sikorski, 1994). Furthermore, roe has high nutritive lipids, particularly phospholipids and long chain unsaturated fatty acids (LCUFAs) (Mahmoud *et al.*, 2008). *cis*-5,8,11,14,17-eicosapentaenoic acid (EPA; C20:5) and cis-4,7,10,13,16,19-docosahexaenoic acid (DHA; C20:6) play an important role in reduction of the risk of coronary heart disease and prevention of Alzheimer's disease and anti-inflammatory disease (Sahena *et al.*, 2009).

Thailand is the world's largest canned tuna producer and exporter. By the year 2012, tuna products were exported totally 0.63 million tons with value of 88,400 million baht (Tangtong, 2013). Tuna roe, a by-product generated from fish processing (approximately 1.5-3.0% of total weight), was generally used as animal feed or for pet food preparation. Due to their benefit on human health, tuna roe can be used as a source of nutrients, especially proteins and lipids for food supplement. Additionally, the selected components, especially protein and protein hydrolysate, can be used as the potential ingredients for food application. Thus, the recovery of those valuable components from tuna roe can increase its value and reduce waste disposal or treatment, thereby lowering environmental pollution. Nevertheless, no information on chemical compositions of roes from skipjack, tongol and bonito, which are the economically important species for tuna canning in Thailand exists. Roe of tuna can serve as the source of protein and lipids. Their protein hydrolysate prepared under the

appropriate condition could possess functional properties and bioactivities, which can be further used as potential additive or processing aid, especially for food emulsion. Roe protein hydrolysate can be used as stabilizer in oil-in-water emulsion and for fish oil microencapsulation, particularly after the modification by phenolic compounds. The information gained will provide the better understanding for maximized use of tuna roe as alternative nutrient or processing aids.

1.2 Review of Literature

1.2.1 Fish roe

Fish roes generally contain 57-75% moisture, 15-28% protein, 3.2-9.5% fat and 1.1-1.4% ash as shown in Table 1. The major components of roe are yolk and egg shell protein (Arukwe and Goksoyr, 2003). Roe is a seasonal product obtained during a short period each year. The biochemical composition of fish eggs is dependent upon a variety of factors including genetic differences (Lanes *et al.*, 2012), broodstock diet (Norambuena *et al.*, 2012), age (Bekhit *et al.*, 2009), spawning period (Yanes-Roca *et al.*, 2009) and environmental parameters such as temperature and salinity (Bobe and Labbé, 2010).

Table 1 Chemical composition of roes from different fish species

Chemical composition	Immature Chum[a] Salmon (*Oncorhynchus keta*)	Mature Chum[a] Salmon (*O. keta*)	Rainbow trout[b] (*O. mykiss*)	Catla[c] *(Catla catla)*
Moisture	55.60	68.50	57.00	61.2
Protein	26.16	17.69	28.50	28.2
lipid	10.59	8.45	9.60	3.9
Ash	1.42	1.11	1.30	1.4

Sources : [a] Bekhit *et al* (2009); [b]Mahmoud *et al.* (2008); Balaswamy *et al*. (2009)

1.2.1.1 Roe protein

The major proteins in fish roe are mainly from egg yolk and egg shell (Arukwe and Goksoyr, 2003) consisting of 11% albumins, 75% ovoglobulin and 13% collagen (Sikorski, 1994). Fish yolk proteins are derived from maternal serum protein or vitellogenin (Vg). Vg as the yolk precursor protein, is a large glycophospholipoprotein having two equal subunits (about 200 kDa) (Lucey, 2009). Vg is synthesized in the liver in response to circulating 17β-estradiol and transported via the circulatory system to developing oocytes in ovaries through a receptor-mediated endocytosis (Mommsen and Walsh, 1988; Palumbo *et al.*, 2007). In ovary, Vg is proteolytically cleaved into yolk proteins including lipovitellins (Lv), phosvitins (Pv) and β–components (β'). Lv characteristically consists of two modules, the N-terminal lipovitellin I (LvI) and the C-terminal lipovitellin II (LvII). In vertebrates, an extra phosvitin (Pv) domain is found between LvI and LvII. Typically, Pv consists of long chains of serine residues interrupted by short stretches of basic amino acids (LaFleur *et al.*, 1995). Vg has been widely studied for its role in fish reproduction and as biomarkers for endocrine disrupting xenobiotics (Hiramatsu *et al.*, 2005). Vg is evolutionarily homologous among a large variety of animals. Degradation of Vg might be due to the activity of cathepsin L and B. The primary degradation products of Vg have a role in regulating oocyte hydration and buoyancy of teleostean eggs (Matsubara *et al.*, 2003). Additionally, function of Vg as fish innate immune has been reported. Vg extracted from carp (*Cyprinus carpio*) exhibited antimicrobial activity by agglutinating gram negative (*Escherichia coli*) and gram positive bacteria (*Staphylococcus aureus*) and binding with macrophage in dose dependent manner (Li *et al.*, 2009) Vg could be isolated and purified using anion exchanger (hydroxylapatite) column, immunoadsorbent column and gel filtration (Amano *et al.*, 2007). Vgs of grey mullet (*Mugil caphalus*) had apparent molecular weight (MW) of 570, 580 and 335 kDa (Amano *et al.*, 2007). Hiramatsu and Hara (1996) characterized Vg and its three egg yolk proteins from Sakhalin tiamen (*Hucho perryi*) using Sepharose 6B chromatography and immonodiffusion for Vg identification and Sephadex G-200 for separating Lv and Pv - β' complex. Vg had an apparent molecular weight of 540 kDa and appeared as a major 240 kDa band in SDS-PAGE,

which resolved into two major bands (165 and 125 kDa) after reduction. The estimated molecular weights of purified Lv, Pv and β' were 330, 23 and 30 kDa, respectively. Riazi *et al.* (1988) isolated egg yolk proteins from rainbow trout (*Salmo gairdneri*) using chromatographic and electrophoretic procedures. Three main components including Lv, Pv and β' with MW of 350, 40 and 30 kDa were obtained from gel filtration. Electrophoretic study revealed that Lv had four subunits with MW of 145-160 kDa and 14-19 kDa (Riazi *et al.*, 1988).

The fish roes are rich in amino acids. Dominant amino acids of fish roes are glutamic acid, followed by leucine, proline, lysine and aspartic acid, while methionine, cysteine, tyrosine and tryptophan are negligible (Bledsoe *et al.*, 2003). Bekhit *et al.* (2009) found that immature and mature chinook salmon roes contained high content of essential amino acid (EAA) such as arginine (5.6-6.5 g/16gN), isoleucine (4.2-6.2 g/16gN), leucine (6.5-7.4 g/16gN), lysine (6.6-6.8 g/16gN), phenylalanine (4.5-5.8 g/16gN) and valine (3.8-6.4 g/16gN). Mature chinook salmon roe also had slightly higher ratio of essential amino acids to non-essential amino acids (EAA/NEAA), compared with immature salmon roe (Bekhit *et al.*, 2009).

A. Lipovitellin (Lv)

Lv or high density lipoprotein (HDL) is the major nutritional resource for embryo growth and is generally found in granule form of lipovitellin-phosvitin complex via phosphocalcic bridge (Anton, 2007). Lv appears to have evolutionary and structural relationships to other lipoproteins such as apolipovitellin, microsomal triglyceride transfer protein and segments of apolipoprotein B (Thompson and Banaszak, 2002). Lv made up of 75-80% proteins and 20-25% lipids (Anton, 2007). Lipid of Lv consists of 65% PL, 30% TG and 5% cholesterol (Cook and Martin, 1969). Lv contains two monomers with equal MW (200 kDa). Each of monomers is globular protein containing a cavity possessing two β-sheets which predominantly constitute hydrophobic amino acids. The hydrophobic amino acid residues in cavity results in the entrapment of lipids, leading to its thermostable properties (Guo *et al.*, 2005). Two sub-groups of Lv separated by ion exchange chromatography are classified as α- and β-HDL (ratio of 1:1.5) (Anton, 2007). The α-HDLs have sialic

acid and phosphorus contents more than that of β-HDLs approximately 6 and 2 times, respectively. Thus, α-HDLs have lower pH than that of β-HDLs. Except for these differences, two types of HDL have a similar content of hydrophobic amino acids (30%) (Anton, 2007). HDLs commonly precipitate in water but are soluble in salt solution with ionic strength above 0.3 M NaCl. Two subunits of Lv have MW of 115 and 31 kDa. The larger subunit has negligible content of phosphate, while the smaller subunit has high phosphate content. Each monomer of HDL is composed of 5 main apoproteins with MW ranging from 35 to 110 kDa (21% of 110 kDa, 16% of 100 kDa, 20% of 80 kDa, 14% of 50 kDa and 28% of 35kDa). Apoproteins of HDL are glycosylated with 0.75% carbohydrate including mannose, galactose, glucosamine and sialic acid (Anton, 2007). Fish Lv is thermostable (Lucey, 2009). Hartling *et al.* (1997) reported that Lv extracted from winter flounder (*Pseudopleuronectes americanus*) is a mixture of heat-stable and heat-labile proteins. Lucey (2009) studied the thermodynamic properties of Lv extracted from ovaries of four pleuronectid species including American plaice, winter flounder, witch flounder and yellowtail flounder. Lvs from four pleuronectid species had high thermostability. Lv plays an essential role in the hydration of eggs in pelagic fish due to its cleavage form (free amino acids), whereas in benthic fish, Lv plays little impact on the hydration of egg (Finn *et al.*, 2002). Furthermore, Lv also aids in the osmoregulation (Reith *et al.*, 2001).

B. Phosvitin

Phosvitin (Pv) is a heavy phosphorylated protein containing high amount of serine residues (approximately 50%). Fish yolk protein had 3-4% Pv, whereas chicken egg yolk protein had 11% Pv (Anton *et al.*, 2007). Hen egg yolk Pv is a mixture of two polypeptides: α-phosvitin (160 kDa) and β- Pv (190 kDa) (Anton *et al.*, 2007). Both Pvs are an aggregate of many different polypeptides: α- Pv contains three or four sub-units of 35 to 40 kDa, whereas β- Pv contains four or five sub-units of 45 kDa. The two Pvs contain 3% (α) and 10% (β) phosphorus, which accounts for about 80% of total protein phosphorus of yolk (Joubert and Cook, 1958). Phosphorus is present as phosphoric acid bound to seryl residues; about 96% of seryl residues are phosphorylated (Anton *et al.*, 2007). For fish egg, phosphorous is a

macromineral important for embryo development and maintenance of the skeletal system (Lanes *et al.*, 2012). In general, fish phosvitin has smaller in size, compared to *Xenopus* and chicken phosvitin (Prakasha *et al.*, 2013). Pv from rainbow trout roes has MW of 20 kDa containing serine, arginine and aspartic acid approximately 57, 15 and 10 residues/molecule, respectively (Suzuki and Suyama, 1985). Negligible aromatic and sulfur-containing amino acids were observed in Pv. Pv from oocytes of Indian freshwater murrel, which was isolated by phenol extraction method, was less than MW of 14 kDa (Prakasha *et al.*, 2013). One molecule of hen egg yolk Pv (40 kDa) has 5 glucosamines, 3 mannoses, 3 galactoses and 2 sialic acid. Major carbohydrates in one molecule of Pv from rainbow trout are 7 glucosamines, 5 mannoses and 3 galactoses. This carbohydrate moiety is bound with asparagine via N-glycosylation. Pv predominantly exists in random coil conformation at neutral pH. Pv is soluble in pure water and easily precipitated with magnesium sulfate. The isoelectric point of Pv is 4.0 (Anton *et al.*, 2007). The conformation of Pv has blocks of phosphoserines in singular way, carrying up to 15 consecutive residues (Figure 1).

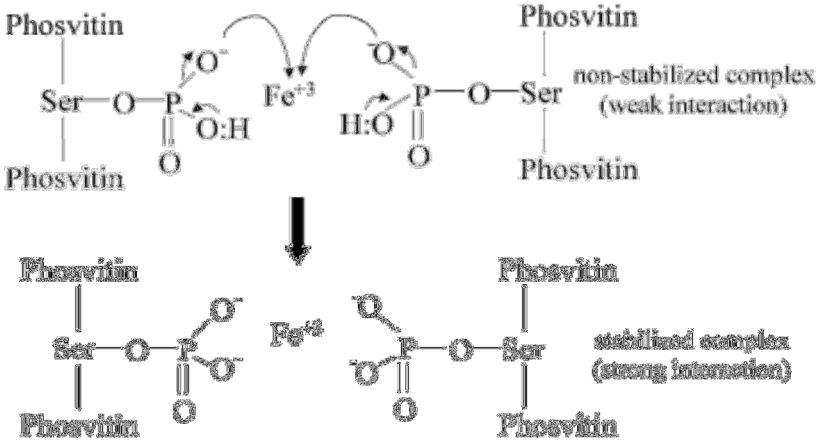


Figure 1 Proposed mechanism for the transition from a weak to strong interaction between ferric ion and phosvitin at pH 3.5. The thin arrows show the direction of the electronic density induction.

Source: Castellani *et al*. (2004)

This unique primary structure of Pv provides the strongest metal chelating agent (Anton *et al.*, 2007; Castellani *et al.*, 2003). Polyanionic character of phosvitin is also known for its capacity to stabilize emulsions (Castellani *et al.*, 2006; Castellani *et al.*, 2005; Guilmineau and Kulozik, 2006) .

(1) Bioactivity of Pv

The metal chelating activity of Pv depends on temperature, pH, ionic strength (Castellani *et al.*, 2004) and type of metal ions (Grizzuti and Perlmann, 1973). One Pv molecule can anchor 113 ions of Mn^{+2} or 120 of Co^{+2} at temperature below 60°C (Grizzuti and Perlmann, 1973). An irreversible thermal transition of Pv is found at 79°C (Anton *et al.*, 2007). At pH 4.5, Pv can interact with Mg^{+2} or Ca^{+2} at level of 40 or 32 ions/molecule, while higher chelating level was obtained at pH 6.5 (103 ions of Mg^{+2} or 127 ions of Ca^{+2}). With varying of pHs (3.0-7.0) and ionic strengths (0.10-0.60), it was found that Pv extracted from isabrown egg exhibited the highest iron binding capacity (115µg of iron/mg of phosvitin) at pH 6.5 and 0.15 M of ionic strength (Castellani *et al.*, 2004). Iron-Pv interaction relies on the methods of ferric ions addition. Direct addition of Pv into a ferric solution, the iron/Pv interaction led to insoluble matter (McCollum *et al.*, 1986; Taborsky, 1991). When ferrous ions are oxidized into ferric ions, it interacts with Pv, rendering a formation of soluble Fe-Pv complexes. Pv also exhibits higher iron binding capacity than that of citrate and nitrilotriacetate. Iron binding capacity indicates the antioxidant activities (Lee *et al.*, 2002; Nakamura *et al.*, 1998) and bactericidal properties (Khan *et al.*, 2000). Lee *et al* (2002) reported that Pv could inhibit lipid oxidation in phosphatidylcholine liposome, pork muscle homogenate and ground pork with dose-dependent manner. The heat treatment resulted in slight reduction of antioxidant activity. Pv was a more effective antioxidant in cooked ground pork (11-39% inhibition), compared with in uncooked ground pork (0-20% inhibition).

(2) Emulsifying property of Pv

Pv has been demonstrated for its emulsifying properties (Castellani *et al.*, 2005; Castellani *et al.*, 2003; Guilmineau and Kulozik, 2006). Due to its

polyanionic character, Pv is also known for its capacity to stabilize emulsions. The high charge density of the non-absorbed part of Pv provides excellent electrostatic repulsion of Pv -coated droplets and excellent action against flocculation and coalescence (Guérin-Dubiard *et al.*, 2002). Emulsifying properties of Pv are affected by several factors (ionic strength, heating temperature, pH, etc.). Ionic strength conditions can affect the aggregated and charged states of proteins, altering thus their emulsifying properties. Castellani *et al.* (2005) studied emulsifying properties of Pv at pH 6 in aggregated and non-aggregated states, at two different ionic strengths (0.05 and 0.15 M). The results indicated that non-aggregated Pv had better emulsifying activity than aggregated Pv. Nonetheless, aggregated Pv stabilized emulsions against coalescence more effectively.

In fish roes, several steps were implemented to separate and purify Pv. Mano and Lipmann (1966) isolated Pv from roes of ling, flounder, cod, shad and salmon. Several steps were used to isolate phosvitins including extraction (using a mixture solution of 0.15 M KCl in 0.025 M $NaHCO_3$/ 0.02 M NaF), precipitation (ammonium sulfate and barium sulfate), centrifugation and dialysis. The obtained crude phosvitins from roes from five fish species were purified using anion exchange chromatography as diethylaminoethyl cellulose (DEAE). Suzuki and Suyama (1985) purified Pv using a DEAE-cellulose column. Pv from rainbow trout egg had MW of 20 kDa with equal molar ratio of serine and phosphate. One molecule of rainbow trout phosvitin contained 57 serine, 15 arginine and 10 aspartic acid and also contained 7 glucosamine, 5 mannose and 3 galactose.

C. Other proteins

Fish roe is presented in *zona radiata* (Zrp) which is sequestered from circulating plasma and formed during oocyte development. Zrp is located in egg shell layer or acellular layer (Arukwe and Goksoyr, 2003). These extracellular coats apparently function to restrict fertilization of eggs to sperm from the same species, to limit fertilization to a single sperm and to protect developing embryos either outside or within the female reproductive tract (Darie *et al.*, 2004). Zrp are derived from choriogenin (CHg) or vitelline envelope (VE). VE from different fish roes such as

rainbow trout, gold fish (*Carrassius auratus*), pipe fish (*Syngnathus scovelli*) and cod were characterized (Darie *et al.*, 2004; Hyllner and Haux, 1995). Three major proteins with MW of 60, 55 and 50 kDa were observed in the VE of rainbow trout. Among three major proteins of VE from rainbow trout, a protein with MW of 50 kDa is only an asparagine-linked glycoprotein, while others were not glycoprotein. Amino composition of VE from 8 species indicated that proline and glutamine/glutamic acid were major, while cysteine was minor (Hyllner and Haux, 1995). Modig *et al.* (2006) reported that acellular envelope of gilthead seabream (*Sparus aurata*) contained 2-4 isoforms of Zrps (ZP1, ZP2, ZP3 and ZPX). ZP2 was not found in teleost fish and ZPX was not found in mammals (Modig *et al.*, 2006).

1.2.1.2 Roe lipid

Fish oils/lipids are well known as a source of polyunsaturated fatty acids (PUFAs), especially docosahexaenoic acid (DHA, 22:6 n-3) and eicosapentaenoic acid (EPA, 20:5 n-3). Commercial fish oils are prepared from flesh and liver. In fish oil, n-3 fatty acids are not only structured in triacylglyceral (TAG), but can be also found as phospholipids (PL) (Tupler *et al.*, 2001), which are major constituents of the cell membranes. The main PL constituents in fish are phosphatidylcholine (PC) (Gierthy *et al.*, 1988) (60–70%) and phosphatidylethanolamine (PE) (20-30%), while minor phospholipids as phosphatidylinositol (PI) and phosphatidylserine (PS) are less abundant. In fish roes, lipids are energy reserves and components of cell bio-membranes. The chemical composition of eggs is usually examined to evaluate egg quality as nutrition requirement for embryonic and larval development. Certain larval stage needed PL for growth and development, while TAG are required as main energy source in egg and yolksac larvae of many fishes (Mukhopadhyay and Ghosh, 2007). The main lipid class is polar lipid (ca. 76% of total lipids), triglycerides, free fatty acids and sterols. Polar lipids contain high levels of PUFAs. Mukhopadhyay and Ghosh (2007) studied lipid content and fatty acid profiles of roes from two different Indian silurid catfish including *Ompok pabda* and *Wallagu attu*. The lipid content of *O. pabda* and *W. attu* roes are about 14.7% and 17.8% (dry basis), respectively. Major lipids were phospholipid (56.8% and 28.6% observed in *O. pabda* and *W. attu* roes, respectively)

and triacylgylcerol (29.5 and 38.4% observed in *O. pabda* and *W. attu* roes, respectively). In general, quantity and compositions of fish lipids vary with species and habitats (Shamsudin and Salimon, 2006).

Fish roes from different species and different maturation stages had high content of n-3 fatty acids approximately 36-43% with low amount of n-6 fatty acids (1.4-6.1%) (Table 2). PUFAs (42-47%) were the main fatty acids in fish roes, especially EPA (7-19%) and DHA (21-29%). Beneficial health effects of PUFAs have been demonstrated as prevention cardiovascular disorder such as arteriosclerosis and myocardial infarction (Lee *et al.*, 2003). The supplementation of n-3 fatty acids could double the membrane DHA concentration during infant period, improve visual activity and decrease the accumulation of body fat (Jørgensen *et al.*, 2001). The n-3 fatty acids play important role in biochemical and functional growth of the normal central nervous system predominantly in brain and retina as evidenced by a high DHA level in term of phospholipids (Jørgensen *et al.*, 2001; Salem *et al.*, 2001). The daily consumption of 30 g of fish can reduce the death rate associated with coronary heart diseases about 50% (Jørgensen *et al.*, 2001).

Table 2 Fatty acid profiles of roe from different fish species

Fatty acid	Immature Alaska[a] Walleye Pollock (*Theragra chalcogramma*)	Mature Chinook[b] salmon (*Oncorhynchus tshawytscha*)	Immature Chinook[b] salmon (*Oncorhynchus tshawytscha*)	Rainbow trout[c] (*Oncorhynchus mykiss*)	Atlantic halibut[d] (*Hippoglossus hippoglossus* L.)
C12:0	ND	0.02±0.04	0.07±0.00	ND	ND
C14:0	2.46±0.04	1.54±0.16	1.35±0.00	3.00±0.10	2.87±0.70
C15:0	0.26±0.00	0.35±0.01	0.34±0.01	ND	ND
C16:0	21.23±0.05	11.65±0.42	12.15±0.23	16.10±0.00	20.30±1.40
C16:1 n9	0.35±0.01	0.01±0.01	0.02±0.02	ND	ND
C16:1 n7	4.78±0.06	4.54±1.05	3.20±0.06	6.20±0.00	3.50±0.70
C16:1 n5	0.28±0.01	0.76±0.07	0.65±0.01	ND	ND
C17:0	ND	0.28±0.02	0.29±0.02	ND	ND
C17:1 n9	1.22±0.05	0.54±0.07	0.46±0.03	ND	ND
C18:0	2.14±0.01	4.32±0.39	3.95±0.17	5.10±0.00	3.70±0.50
C18:1 n11	0.54±0.01	ND	ND	ND	ND
C18:1 n9	11.56±0.13	22.33±2.03	19.68±0.34	15.6±0.10	8.70 ± 1.00
C18:1 n7	5.93±0.02	3.25±0.46	2.66±0.04	4.30±0.20	ND
C18:1 n5	0.21±0.00	ND	ND	ND	ND

Sources: [a]Bechtel *et al.*(2007); [b]Bekhit et al. (2009); [c]Mahmoud *et al.* (2008); [d]Mommens *et al.* (2013)

ND: not detectable

Table 2 Fatty acid profiles of roe from different fish species (cont.)

Fatty acid	Immature Alaska[a] Walleye Pollock (*Theragra chalcogramma*)	Mature Chinook[b] salmon (*Oncorhynchus tshawytscha*)	Immature Chinook[b] salmon (*Oncorhynchus tshawytscha*)	Rainbow trout[c] (*Oncorhynchus mykiss*)	Atlantic halibut[d] (*Hippoglossus hippoglossus* L.)
C18:2	ND	3.42±0.68	2.53±0.02	ND	ND
C18:2 n6 *cis*	0.57±0.02	ND	ND	4.20±0.00	4.00±1.50
C18:3 n4	0.21±0.00	ND	ND	ND	ND
C18:3 n3	0.21±0.00	0.60±0.08	0.50±0.00	ND	0.50±0.10
C18:3 n6	ND	0.08±0.00	0.05±0.04	ND	0.10±0.00
C18:4 n3	0.69±0.01	0.53±0.01	0.53±0.01	ND	0.30±0.10
C20:0	ND	ND	ND	ND	0.10±0.10
C20:1	ND	2.25±0.19	2.51±0.06	ND	ND
C20:1 n11	1.32±0.03	ND	ND	ND	ND
C20:1 n9	1.07±0.00	ND	ND	3.40±0.00	2.80±1.30
C20:1 n7	0.21±0.02	ND	ND	ND	0.30±0.10
C20:2 n6	ND	ND	ND	ND	0.60±0.20
C20:3 n6	ND	ND	ND	ND	0.10±0.00
C20:4 n6	ND	ND	ND	ND	1.40±0.40

Sources: [a]Bechtel *et al.*(2007); [b]Bekhit et al. (2009); [c]Mahmoud *et al.* (2008); [d]Mommens *et al.* (2013)

ND: not detectable

Table 2 Fatty acid profiles of roe from different fish species (cont.)

Fatty acid	Immature Alaskaa Walleye Pollock (Theragra chalcogramma)	Mature Chinookb salmon (Oncorhynchus tshawytscha)	Immature Chinookb salmon (Oncorhynchus tshawytscha)	Rainbow troutc (Oncorhynchus mykiss)	Atlantic halibutd (Hippoglossus hippoglossus L.)
C20:4 n3	ND	ND	ND	ND	0.40±0.10
C20:5 n3	ND	ND	ND	ND	12.50±1.60
C22:1 n9	ND	ND	ND	ND	1.10±0.80
C22:1 n7	0.47±0.01	ND	ND	ND	ND
C22:5 n3	1.47±0.01	3.85±0.03	3.79±0.04	4.80±0.00	1.40±0.20
C22:6 n3	21.17±0.28	24.32±3.70	29.06±0.15	24.00±0.30	31.20±1.50
C24:0	ND	ND	0.01±0.02	ND	ND
C24:1 n9	ND	0.14±0.14	0.35±0.05	ND	0.70±0.20
SFA	26.10±0.03	18.33±0.41	18.18±0.39	24.20±0.10	26.90±2.60
MUFA	28.76±0.33	33.96±3.34	29.55±0.45	29.40±0.20	19.60±4.40
PUFA	44.52±0.34	42.65±3.65	47.27±0.20	46.40±0.30	53.00±5.90
SFA/PUFA ratio	0.59±0.01	0.43±0.012	0.38±0.017	0.52±0.03	0.51±0.04
n -3	42.87±0.36	36.29±3.82	41.16±0.15	40.30±0.30	46.30±3.50
n -6	1.44±0.02	2.95±0.51	3.58±0.10	6.10±0.00	7.10±1.90
n-3/n-6 ratio	29.75±0.68	12.41±0.76	11.50±0.32	6.61±0.10	6.52±1.84

Sources: [a]Bechtel *et al.*(2007); [b]Bekhit *et al.* (2009); [c]Mahmoud *et al.* (2008); [d]Mommens *et al.* (2013)

ND: not detectable

1.2.2 Protein hydrolysate

Protein hydrolysates are generally prepared from by-products generated from fish- processing. Protein hydrolysates have been obtained by different methods such as acid hydrolysis (Dimitrijev-Dwyer *et al.*, 2012) and enzymatic hydrolysis (Ahn *et al.*, 2010). Use of proteolytic enzymes is an effective approach for improving functional properties of food proteins, without losing their nutritional value. The hydrolytic process and reaction conditions differ for different substrates and enzymes, which also depend on the properties desired for the hydrolysates. Fish protein hydrolysates have been reported to exhibit antioxidative properties, antihypertensive activity (Lee *et al.*, 2010), immunomodulatory activity (Hou *et al.*, 2012) and antiproliferative activity (Hsu *et al.*, 2011). Fish protein hydrolysates are potentially used in many applications including animal feeds (Zheng *et al.*, 2012), foods (Kadam and Prabhasankar, 2010) or nitrogen source of microbial growth media (Klompong *et al.*, 2012; Safari *et al.*, 2012). Additionally, fish protein hydrolysate constitutes a source of health beneficial molecules such as secretagogues, calciotropic hormones and growth factors (Ravallec *et al.*, 2013). The enzymatic hydrolysis also improved functional properties of protein (Sarmadi and Ismail, 2010). The cleavage of protein leads to unfold protein and exposure of hydrophobic region to aqueous phase. Resulting peptides can therefore be different with parent molecule and possibly possess new nutrition, functional and biological properties (Gbogouri *et al.*, 2004).

1.2.2.1 Bioactivity

Enzymatic hydrolysis of food proteins is an efficient way to recover potential bioactive peptides. Hydrolysates from different fish species have been reported for bioactivity including antioxidative properties, therapeutic treatment, ACE-I inhibitory activity and Ca- and/or Fe-binding capacity. The enzymatic cleavage further rendered low molecular weight peptides, which are appropriate for the use as microbial growth media. Fish protein hydrolysates can potentially applied in nutritional or pharmaceutical applications (Wergedahl *et al.*, 2004).

A. Antioxidative properties

Hydrolysates derived from fish proteins have the ability of exerting potent antioxidative activities in different oxidative systems. Batista *et al.* (2010) prepared hydrolysate from black scabbardfish (*Aphanopus carbo*) by-products using Protamex. At 57% degree of hydrolysis (DH), the hydrolysate had peptides with MW lower than 1000 Da. Hydrolysate of black scabbardfish showed antioxidative activities including 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical-scavenging activity, reducing power and hydroxyl scavenging activity. Klompong *et al*. (2007) studied antioxidative activities of protein hydrolysates from yellow stripe trevally (*Selaroides leptolepis*) meat using Alcalase 2.4L (HA) and Flavourzyme 500L (HF). As the DH increased, DPPH radical-scavenging activity and reducing power of HA decreased ($p < 0.05$), but no differences were observed for HF (Klompong *et al.*, 2007). Galla *et al* (2012) prepared hydrolysates from roe protein concentrates of snakehead murrel (*Channa striatus*) and rohu (*Labeo rohita*) using 1% Alcalase. Roe protein hydrolysate from snakehead murrel (28.41%DH) showed superior DPPH radical scavenging activity and ferric reducing power to rohu counterpart (18.85%DH). Roe protein hydrolysate of both species possessed antioxidative activities, which varied depending on concentration used. Dong *et al.* (2008) prepared protein hydrolysate from silver carp (*Hypophthalmichthys molitrix*) using Alcalase and Flavourzyme. Result indicated that Alcalase was much more specific in hydrolysis of defatted meat of silver carp than did Flavourzyme, thus lowering molecular weight of peptides generated with superior antioxidative activities including hydroxyl radical scavenging activity, metal chelating activity and linoleic acid peroxidation. The antioxidative activity of Alcalase-hydrolyzed protein at 1.5 and 2 h of hydrolysis time was comparable to that of α-tocopherol in emulsion system and carnosine in the 2-deoxyribose oxidation system. The lower MW of hydrolysate is suggested to possess stronger Fe^{2+} chelation ability (Dong *et al.*, 2008). Presumably, the increase in chelating activity of hydrolysates might be due to the cleavage of protein, resulting in an increased amount of carboxylic groups (COO^-) and amino groups (NH_2^+) in branches of the acidic and basic amino acids. Consequently,

antioxidative activities of hydrolysate are governed by molecular weight, degree of hydrolysis (DH) and hydrolysis time, which vary with specificity of enzyme used.

B. Therapeutic properties

The hydrolysis process could be further produced various peptides bearing a structural resemblance to hormones. Picot *et al*. (2006) reported that hydrolysate prepared from three blue whiting, three cod, three plaice and one salmon hydrolysates exhibited antiproliferative activity on 2 human breast cancer cell lines grown *in vitro*. Ravallec-Plé *et al.* (2001) studied the bioactivity of Alcalase-hydrolyzed peptide of cooked wastes of sardine (*Sardina pilchardus*) by means of radioimmunoassays and mitogenic and radioreceptor assays. It indicated that molecules related to secretagogue peptides, growth factors and calcitonin gene-related peptide (CGRP) were presented in the hydrolysates. The calcitonin-gene related peptide (CGRP) is a 37-residue peptide widely distributed in the central nervous system and peripheral nerves (Slizyte *et al.*, 2009). Secretagogue activities in AR4-2J pancreatic tumor cells were also found in Alcalase-hydrolyzed peptides prepared from cod (*Gadus morhua*) and shrimp (*Penaeus aztecus*) (Ravallec-Plé and Van Wormhoudt, 2003). Peptides of enzymatically hydrolyzed fish muscle showed anticoagulant and antiplatelet properties tested *in vitro* and these results suggested the capability of fish peptides to inhibit coagulation factors in the intrinsic pathway of coagulation (Rajapakse *et al.*, 2005a).

C. ACE-inhibitory activites

Recently, it has been demonstrated that protein hydrolysates exhibiting antioxidative potential may also contain peptides with other biological activities, such as angiotensin-I-converting enzyme (ACE)-inhibitory activity (Bougatef *et al.*, 2008; Samaranayaka *et al.*, 2010). Angiotensin-I-converting enzyme (ACE) plays an important role in the regulation of blood pressure. Inhibition of ACE is considered to be a useful therapeutic approach in the treatment of hypertension. Natural ACE inhibitors as alternatives to synthetic drugs are of great interest to prevent several side effects (Wijesekara and Kim, 2010). ACE inhibitory activities of protein hydrolysates

prepared from heads and viscera of sardinelle (*Sardinella aurita*) by treatment with various proteases were examined by Bougatef *et al.* (2008). Alcalase, chymotrypsin, crude enzyme preparations from *Bacillus licheniformis* NH1 and *Aspergillus clavatus* ES1 and crude enzyme extract from sardine (*Sardina pilchardus*) viscera were used to hydrolyze protein of sardinelle head and viscera. All resulting hydrolysates exhibited inhibitory activity towards ACE. The highest ACE inhibitory activity (63.2 ± 1.5% at 2 mg/mL) was obtained from hydrolysate produced by alkaline protease extracted from sardine viscera. Size exclusion using a Sephadex G-25 could separate sardine Alkaline protease-hydrolysate into eight major fractions (P_1-P_8). Fraction P_4 was found to display a high ACE inhibitory activity with IC_{50} values for ACE inhibitory activities of approximately 0.81±0.013 mg/mL. The amino acid analysis by GC/MS showed that P_4 was rich in phenylalanine, arginine, glycine, leucine, methionine, histidine and tyrosine. The ACE inhibitory activity of the fraction P_4 after digestion by pepsin, trypsin and mixture of trypsin and chymotrypsin were 73.5, 73.3 and 71.6%, respectively, and that of the control was 74.5%, indicating the resistance to digestion by in gastrointestinal tract (Bougatef *et al.*, 2008). Many studies have shown that peptides with high potent inhibitory activity toward ACE have Trp, Phe, Tyr, or Pro at their C-terminal and branched aliphatic amino acid at the N-terminal (Li *et al.*, 2004). Lee *et al.* (2010) reported that amino acid sequence of the pepsin-hydrolyzed peptide of tuna frame (PTFP) with the highest ACE I inhibitory activity was Gly-Asp-Leu-Gly-Lys-Thr-Thr-Thr-Val-Ser-Asn-Trp-Ser-Pro-Pro-Lys-Try-Lys-Asp-Thr-Pro (MW of 2,482 Da, IC_{50}: 11.28 μm). Lineweaver–Burk plots suggest that PTFP plays as a non-competitive inhibitor against ACE. Antihypertensive effect in spontaneously hypertensive rats (SHR) also revealed that oral administration of PTFP can decrease systolic blood pressure significantly. Increasing hydrolysis time causes the increment of ACE- inhibitory activities and also degree of hydrolysis. The antihypertensive peptides isolated from bonito fish hydrolysate product, were smaller than the initial one. ACE inhibitory activity of antihypertensive peptide was 8-fold higher, compared with the initial peptide (Jung *et al.*, 2006b).

D. Ion-binding properties

The use of enzymatic hydrolysis also provides small peptides with high affinity to calcium and iron, particularly phosphopeptides (Jung *et al.*, 2006b; Jung *et al.*, 2005). Phosphopeptide can be prepared and isolated from several sources including milk protein, fish bone, egg and fish roe, etc. The recovery of those peptides could be achieved by using hydroxyapatite affinity chromatography. Caseinophosphopeptides (CPP), phosphorylated peptides, are known to exert an effect on calcium bioavailability and other minerals because the highly anionic character of CPP allows them to form soluble complexes with calcium (Kim *et al.*, 2007a). Biological functions of CPP are not only to promote Ca-uptake and bone calcification, but are also able to chelate transitional metals (iron, copper and zinc) (Díaz and Decker, 2004). CPP containing the highly phosphorylated polar residues was identified as SerP-SerP-SerP-Glu-Glu. Díaz and Decker (2004) suggested that phosphoresyl residues play a vital role in chelation of Ca and also transitional metal ions. The enzymatic hydrolysis could generate osteoclasts and osteoblasts, which have active roles in bone resorption and deposition, respectively. Since CPP has been reported to increase the absorption of calcium, iron and zinc. Thus CPP is potentially applied in food as enhancers of mineral bioavailability. Phosphopeptides rich in phosphoseryl residues were also obtained by hydrolysis egg Pv by tryptic enzyme (Choi *et al.*, 2005). The resulting egg Pv peptide was examined for its efficiency in enhancing Ca absorption and accumulation in bones of Sprague Dawley rats. The rates of intestinal Ca absorption and its accumulation in bones were significantly higher in the groups, in which phosvitin peptides were added in diets, with concentrations of 0.125–0.5%, which were equivalent amounts of 25–100% of the Ca in the diets (Choi et al., 2005). The skeleton or frame discarded from fish processing as frames and backbones of hoki (*Johnius belengerii*) and Alaska pollock (*Theragra chalcogramma*) has been studied for the availabity of phosphopeptide and its bioactivity by Jung *et al*. (Jung *et al.*, 2006a; 2005). The bone matrice are basically made up of two phases, an organic and an inorganic. The major organic compounds are collagen, followed by noncollagenous protein such as osteocalcin, osteopontin, osteonectin, fibronectin, thrombospondin, proteoglycan and growth factors (Jung *et*

al., 2006a). Crude enzyme from intestine of bluefin tuna (*Thunnus thynnus*) (TICE) was employed in the preparation of phosphopeptide from hoki frame in comparison with commercial protease. The use of TICE liberated total bone hydrolysate approximately 32.12% of total bone slightly lower than did commercial collagenase (32.7%). Protein content of TICE hydrolysate was 21.9% of total soluble hydrolysate containing 13.4% collagen and 10.3% non-collagenous protein, while negligible content of non-collagenous protein (2.13%) was markedly found in collagenase-hydrolyzed peptide. After isolation by hydroxyapatite and gel filtration columns, the resulting fish bone phosphopeptides (FBP) were characterized. Peptides were composed of 23.6% of phosphorus with MW of 3.5 kDa. Gly, Thr, Glx, Ala, Asx, Ser, Hyp and Arg were found as major amino acids in FBP. Gly Hyp and Pro are known as typical amino acids of collagen and its derivatives (Mendis *et al.*, 2005). The Ca-binding capacity of TICE hydrolysate was higher than that of hydrolysate produced by collagenase, therefore the phosphoproteins which had high affinity for Ca were assumably derived from non-collagenous materials in the bone. Nine commercial proteases (Protamex, kojizyme, neutrase, Flavourzyme, pepsin, Alcalase, protease, trpsin and α-chymotrypsin) were applied in preparation of high Ca-binding affinity hydrolysate from Alaska pollock back bone (Jung *et al.*, 2006a). The highest hydrolysis efficiency was attained in the presence of pepsin (87% w/w of total backbone) followed by protease (68%) and α-chymotrypsin (60%), respectively. The different Ca affinity of each hydrolysates prepared using different enzymes used was obtained. The highest content of soluble calcium could be liberated from Alaska pollack backbone by pepsin under the optimal condition (enzyme/substrate: 1/100; substrate concentration: 1%; optimal pH: 2.0; optimal temperature: 37 °C). Amino acid sequence of pepsinolytic hydrolysate was Val-Leu-Ser-Gly-Gly-Thr-Thr-Met-Ala-Met-Tyr-Thr-Leu-Val (1442 Da). *In vitro* assay elucidated that TBP can solubilize a similar content of calcium with casein phosphopeptide (CPP) (Jung *et al.*, 2006a). As reported by Jiang and Mine (2000), ca-binding phosphoproteins such as osteocalcin, phosvitin and casein phosphoprotein, mainly consist of Ser, Thr, Ala and Tyr residues. Thus protein containing Ser, Thr, Ala and Tyr residues phosphorylated or bound to Ca could be used for hydrolysate preparation with calcium binding ability.

1.2.2.2 Bioactive peptides

Bioactive peptides are liberated during hydrolysis and they vary in structural, compositional and sequential properties and may exhibit various bioactivities such as antioxidative (Rajapakse *et al.*, 2005b), antihypertensive (Ghassem *et al.*, 2011) and immunomodulatory effects (Hou *et al.*, 2012). The amino acid sequences of hydrolysate containing bioactive peptides have been characterized as shown in Table 3. Amino acid sequence of peptide containing Tyr, Met, His, Lys and Trp are generally accepted as antioxidants, despite their occasionally pro-oxidative effects. The antioxidative activity of His-containing peptides was attributed to the proton-donation ability of the His imidazole group (Li *et al.*, 2007). Furthermore, the peptides contained His residues at the second residue in the sequence, such as Ala-His, Val-His-His and Val-His-His-Ala-Asn-Glu-Asn from egg white albumin possessed antioxidative acitivty (Tsuge *et al.*, 1991).

Table 3 Bioactive peptides and their amino acid sequences of fish hydrolysates produced by enzymatic hydrolysis

Samples	Enzymes used	Amino acid sequences	Bioactivities	References
Giant squid (*Dosidicus gigas*) muscle protein	Trypsin	Asn-Ala-Asp-Phe-Gly-Leu-Asn-Gly-Leu-Glu-Gly-Leu-Ala **(1307 Da)** Asn-Gly-Leu-Glu-Gly-Leu-Lys **(747 Da)**	Radical scanvenging activity	(Rajapakse *et al.*, 2005b)
Hoki (*Johnlus belengerii*) skin gelatin	Trypsin	His-Gly-Pro-Leu-Gly-Pro-Leu (**797 Da**)	Radical scanvenging activity	(Mendis *et al.*, 2005)
Tuna backbone protein	Pepsin	Val-Lys-Ala-Gly-Phe-Ala-Trp-Thr-Ala-Asn-Glu-Glu-Leu-Ser **(1519 Da)**	Radicals (DPPH, hydroxyl and superoxide) scavenging activity	(Je *et al.*, 2007)
Rotifer (*Brachionus rotundiformis*)	Pepsin	Leu-Leu-Gly-Pro-Gly-Leu-Thr-Asn-His-Ala **(1076 Da)** Asp-Leu-Gly-Leu-Gly-Leu-Pro-Gly-Ala-His **(1033 Da)**	DPPH radical scavenging activity	(Byun *et al.*, 2009)
Cooking juice from long tail tuna (*Thunnus tonggol)*	Orientase	Pro-Val-Ser-His-Asp-His-Ala-Pro-Glu-Tyr (**1305 Da**) Pro-Ser-Asp-His-Asp-His-Glu (**938 Da**) Val-His-Asp-Tyr (**584 Da**)	DPPH radical scavenging activity	(Hsu *et al.*, 2009)
Sardinelle (*Sardinellaaurita*)	Crude enzyme extract from sardine (*Sardinapilchardus*)	Leu-His-Tyr (**431 Da**) Gly-Ala-Leu-Ala-Ala-His (**538 Da**) Gly-Ala-Trp-Ala (**403 Da**) Leu-Ala-Arg-Leu (**471 Da**)	DPPH radical scavenging activity	(Bougatef *et al.*, 2010)

Table 3 Bioactive peptides and their amino acid sequences of fish hydrolysates produced by enzymatic hydrolysis (cont.)

Samples	Enzymes used	Amino acid sequences	Bioactivities	References
Dark muscle of long tail tuna (*Thunnus tonggol)*	Orientase protease XXIII	Leu–Pro–Thr–Ser–Glu–Ala–Ala–Lys–Tyr (**978 Da**) Pro–Met–Asp–Tyr–Met–Val–Thr (**756 Da**)	DPPH radical scavenging activity	(Hsu, 2010)
Horse mackerel (*Magalaspis cordyla*) skin	Pepsin	Asn-His-Arg-Tyr-Asp-Arg (**856 Da**)	Radicals (DPPH and hydroxyl) scavenging activity	(Kumar *et al.*, 2012)
Croaker (*Otolithes ruber*)	Pepsin	Gly-Asn-Arg-Gly-Phe-Ala-Cys-Arg-His-Ala (**1101.5 Da**)	Radicals (DPPH and hydroxyl) scavenging activity	(Kumar *et al.*, 2012)
Tilapia (*Oreochromis niloticus*) skin gelatin	Multifect neutral and properase E	Glu-Gly-Leu (**317 Da**) Tyr-Gly-Asp-Glu-Tyr (**645 Da**)	Radicals (DPPH, hydroxyl and superoxide anion) scanvenging activity	(Zhang *et al.*, 2012)
Frame, bone and skin from threadfin beam	*Virgibacillus* sp. SK33 proteinase	Phe-Leu-Gly-Ser-Phe-Leu-Tyr-Glu-Tyr-Ser-Arg (**1382 Da**)	Radicals (ABTS and cellular) scavenging activity	(Wiriyaphan *et al.*, 2013)
Spotless smoothhound (*Mustelus griseus*) muscle	Papai n	Gly-Ala-Ala (**217 Da**) Gly-Phe-Val-Gly (**378 Da**) Gly-Ile-Ile-Ser-His-Arg (**682 Da**) Glu-Leu-Leu-Ile (**486 Da**) Lys-Phe-Pro-Glu (**519 Da**)	Radicals (ABTS, hydroxyl and superoxide anion) scanvenging activity	(Wang *et al.*, 2014a)

Table 3 Bioactive peptides and their amino acid sequences of fish hydrolysates produced by enzymatic hydrolysis (cont.)

Samples	Enzymes used	Amino acid sequences	Bioactivities	References
Round scad (*Decapterus maruadsi*) muscle	Alcalase	His-Asp-His-Pro-Val-Cys (**706.8 Da**) His-Glu-Lys-Val-Cys (**614.7 Da**)	Radicals (DPPH and superoxide anion) scanvenging activity	(Jiang *et al.*, 2014)
Pectoral fin of salmon	Pepsin	Phe-Leu-Asn-Glu-Phe-Leu-His-Val (**1018 Da**)	Radicals (DPPH and ABTS) scanvenging activity, ferric reducing power, prevention of hydroxyl radical-induced DNA damage and hydrogen peroxide-induced hepatic damage in Chang liver cells.	(Ahn *et al.*, 2014)
Oyster (*Crassostrea talienwhanensis Crosse*)	Subtilisin	Pro-Val-Met-Gly-Asp (**518 Da**) Gln-His-Gly-Val (**440 Da**)	Radicals (DPPH and Hydroxyl) scanvenging activity	(Wang *et al.*, 2014b)
Shrimp (*Acetes chinensis*)	Alcalase	Ile-Phe-Val-Pro-Ala-Phe **(783 Da)**,	ACE inhibitory activity	(He *et al.*, 2006)
Shark meat	Protease of *Bacillus* sp. SM98011	Lys–Pro–Pro–Glu–Thr–Val **(815 Da)**, Tyr–Leu–Leu–Phe **(609 Da)**, Phe–Cys–Val–Leu–Arg–Pro **(824 Da)**, Ile–Phe–Val–Pro–Ala–Phe **(783 Da)** Ala–Phe–Leu **(385 Da)**	ACE inhibitory activity	(Wu *et al.*, 2008)

Table 3 Bioactive peptides and their amino acid sequences of fish hydrolysates produced by enzymatic hydrolysis (cont.)

Samples	Enzymes used	Amino acid sequences	Bioactivities	References
Tuna frame protein	Pepsin	Gly-Asp-Leu-Gly-Lys-Thr-Thr-Thr-Val-Ser-Asn-Trp-Ser-Pro-Pro-Lys-Try-Lys-Asp-Thr-Pro (**2482 Da**)	ACE inhibitory activity	(Lee *et al.*, 2010)
Cuttlefish (*Sepia officinalis*) muscle	Crude protease extract from the hepatopancreas of cuttlefish	Val-Tyr-Ala-Pro (**448 Da**) Val-Ile-Ile-Phe (**491 Da**) Met-Ala-Trp (**407 Da**)	ACE inhibitory activity	(Balti *et al.*, 2010)
Haruan (*Channa striatus*) myofibrillar protein	Thermolysin	Val-Pro-Ala-Ala-Pro-Pro-Lys (**791 Da**) Asn-Gly-Thr-Trp-Phe-Glu-Pro-Pro (**1085 Da**)	ACE inhibitory activity	(Ghassem *et al.*, 2011)
Grass carp (*Ctenopharyngodon idella*)	Alcalase	Val-Ala-Pro (**285 Da**)	ACE inhibitory activity	(Chen *et al.*, 2012)
Lizard fish (*Saurida elongata*) muscle	Neutral protease	Ser-Pro-Arg-Cys-Arg (**618 Da**)	ACE inhibitory activity	(Wu *et al.*, 2012)
Snakehead fish sarcoplasmic protein	Alcalase	Leu-Tyr-Pro-Pro-Pro (**585 Da**) Tyr-Ser-Met-Tyr-Pro-Pro (**756 Da**)	ACE inhibitory activity	(Ghassem *et al.*, 2013)
Gelatin of skate (*Okamejei kenojei*) skin	Alcalase and protease	Met-Val-Gly-Ser-Ala-Pro-Gly-Val-Leu (**829 Da**) Leu-Gly-Pro-Leu-Gly-His-Asp (**720 Da**)	ACE inhibitory activity	(Ngo *et al.*, 2014)

Table 3 Bioactive peptides and their amino acid sequences of fish hydrolysates produced by enzymatic hydrolysis (cont.)

Samples	Enzymes used	Amino acid sequences	Bioactivities	References
Alaska 25ollock (*Theragra chalcogramma*) backbone	Crude enzyme from intestine of bluefin tuna	Val-Leu-Ser-Gly-Gly-Thr-Thr-Met-Ala-Met-Tyr-Thr-Leu-Val **(1442 Da)**	Ca-binding capacity	(Jung *et al.*, 2006a)
Shrimp processing byproducts	Trypsin	Thr-Cys-His (**359 Da**)	Ca-binding capacity	(Huang *et al.*, 2011)
Tilapia (*Oreochromis niloticus*) meat	Alcalase	Trp-Glu-Trp-Leu-His-Tyr-Trp	Ca-binding capacity	(Charoenphun et al., 2012)
Tilapia (*Oreochromis niloticus*) scale	Trypsin and Flavourzyme	Asp-Gly-Asp-Asp-Gly-Glu-Ala-Gly-Lys-Ile-Gly (**1033 Da**)	Ca-binding capacity	(Chen *et al.*, 2014)
Alaska pollock (*Theragra chalcogramma*) frame	Trypsin	Asn-Gly-Met-Thr-Tyr (**585 Da**) Asn-Gly-Leu-Ala-Pro (**471 Da**) Trp-Thr (**305 Da**)	Immunomodulating activities	(Hou *et al.*, 2012)

Kim *et al.* (2007b) prepared antioxidant peptide from hoki (*Johnius belengerii*) frame protein using six proteases including pepsin, trypsin, papain, α-chymotrypsin, Alcalase and Neutrase. Among of six proteases, pepsin was found to produce effective bioactive peptide with scavenging activity toward different radicals (DPPH, hydroxyl, superoxide and peroxyl). The amino acid sequence of pepsin hydrolyzed-peptide with MW of 1,801 Da was Glu-Ser-Thr-Val-Pro-Glu-Arg-Thr-His-Pro-Ala-Cys-Pro-Asp-Phe-Asn. This purified peptide further decreased radical-mediated cytotoxicity on MRC-5 cells and protected free-radical-induced DNA damage (Kim *et al.*, 2007b). Hsu *et al.* (2009) prepared antioxidative peptides from tuna cooking juice hydrolysate by orientase. Three major antioxidative peptides are Pro-Val-Ser-His-Asp-His-Ala-Pro-Glu-Tyr (1,305 Da), Pro-Ser-Asp-His-Asp-His-Glu (938 Da) and Val-His-Asp-Tyr (584 Da), respectively. Hydrolysate of dark muscle byproduct was also proprepared using two commercial enzymes (orientase or protease XXIII) (Hsu, 2010). Hydrolysates possessing the highest antioxidative activity were obtained when hydrolysis times of 60 and 120 min were used for orientase and protease XXIII, respectively. After purification by Sephadex G-25 gel filtration chromatography and two-step high-performance liquid chromatography, amino acid sequences of the two antioxidative peptides from orientase- and protease XXIII-hydrolysates were Leu–Pro–Thr–Ser–Glu–Ala–Ala–Lys–Tyr (978 Da) and Pro–Met–Asp–Tyr–Met–Val–Thr (756 Da), respectively.

1.2.2.3 Microbial media

After hydrolysis, protein hydrolysates was rich in low molecular weight peptides and free amino acids, which can be used as an excellent nitrogen source for microbial growth (Klompong *et al.*, 2009a; Safari *et al.*, 2012; Vázquez *et al.*, 2008). Protein hydrolysates suitable for culturing microorganisms generally consist of organic and inorganic materials (Kurbanoglu and Algur, 2004) such as calcium, sodium, magnesium, potassium, phosphorous, iron, copper, manganese, zinc and sulfur which are required for microbial growth (Kurbanoglu and Algur, 2004). Fish wastes have great potential to be used as nutrient sources for bacterial growth, because cell production is largely controlled by the cost related to the growth media and the use of by-products can reduce the production cost. Many pretreatments are

applied to produce fish-based media for bacterial growth. The available methods include heat (Rebah *et al.*, 2008), enzymatic and chemical (Beaulieu *et al.*, 2009). Fish byproducts from several species such as trout, swordfish, tuna or cephalopod were used to prepare hydrolysate for culture media purpose (Vázquez *et al.*, 2008). Vázquez *et al.* (2008) found a great vatiety of microbial responses with different fish species used as peptone source. Peptones from eviscerates of the fishing byproducts with a slight supplement of yeast extract, showed excellent capability for promoting growth and the characteristic bioproductions (lactic and acetic acids) of lactic acid bacteria. The cuture of *Lactobacillus plantarum*, herterofermetative bacteria, generated the highest mass production of acetic acid and lactic acid, when cultured by using ray or shark protein hydrolysate as growth media and higher than that of MRS. Shark peptone was also superior in biomass productivity in culture of *Lactobacillus buchneri* than MRS and other marine hydrolysate peptones (Vázquez *et al.*, 2008). Safari *et al.* (2012) prepared hydrolysates from yellowfin tuna (*Thunnus albacares*) head using Alcalase or protamex. Resulting hydrolysates were used as peptone which more effectively promoted the growth of lactic acid bacteria than commercial MRS media. Between two different enzymes, Alcalase-hydrolyzed protein with higher DH rendered the higher microbial growth than protamex-hydrolyzed protein (Safari *et al.*, 2012). Klompong *et al.* (2009a) prepared protein hydrolysate from mince of yellow stripe trevally, a low market-value fish using Flavourzyme to obtain a DH of 25% (HF25). HF25 was characterized and examined for the use as culture media in comparison with commercial Bacto Peptone. HF25 had the higher contents of ash (45.73%), lipid (0.77%) and moisture (4.34%) but lower protein content (42.11%) than did Bacto Peptone. HF25 contained a higher amount of essential amino acids (44.05%) than did Bacto Peptone (19.34%). HF25 and Bacto Peptone consisted of several minerals at varying levels and had an excellent solubility over a wide pH range. The differences in amino acid profile between HF25 and Bacto Peptone might be caused by the existing differences in raw material, pretreatment, hydrolysis conditions such as pH and temperature of hydrolysis process and enzyme type used. HF25 showed the higher bacterial productivity ratio than did Bacto Peptone. When HF25 and commercial Bacto Peptone were used as microbial media to determine microbial load of environmental and food samples and pathogenic bacteria, HF25

generally exhibited similar potential in culturing those microorganisms. Thus, the conversion of low market value species and fish byproducts to fish protein hydrolysate, can provide nitrogenous substrate for microbial growth.

1.2.2.4 Functional properties

Functional properties of protein can be improved by enzymatic hydrolysis under controlled conditions (Sarmadi and Ismail, 2010). Enzymatic hydrolysis results in the change of surface hydrophobicity, molecular weight distribution, polar group and emulsifying properties of the resulting hydrolysate (Aewsiri *et al.*, 2009). The characteristics of hydrolysate directly have the impact on the functional properties and their application in food system (Choi *et al.*, 2009; Klompong *et al.*, 2007). Generally, hydrolysate has an excellent solubility at high degree of hydrolysis (Gbogouri *et al.*, 2004). Level of hydrolysis varys according to protein source, specificity of enzyme used, pH, temperature, time and enzyme to substrate level (Bhaskar *et al.*, 2008).

A. Solubility

The solubility is an important functional properties of hydrolysate required for liquid foods, while emulsion and water adsorption is important for semisolid foods (Bhaskar *et al.*, 2008). High solubility of fish protein hydrolysate over a wide range of pH influences the functional properties of the resulting hydrolysate such as foaming and emulsifying properties and is a substantially useful characteristic for many food applications (Klompong *et al.*, 2007). Giménez *et al.* (2009) reported that the use of Alcalase in hydrolysis of squid and sole skin-gelatins resulted in protein solubility above 95%. Klompong *et al.* (2009a) reported that the use of Alcalase and Flavourzyme for hydrolyzing yellow stripe trevally (*Selaroides leptolepis*) meat, could increase protein solubility to above 85% over a wide pH range (2-12). The use of Alcalase in protein hydrolysis of Pacific whiting (*Merluccius productus*) resulted in the complete solubility at various pHs (4.0, 7.0 and 10.0) (Pacheco-Aguilar *et al.*, 2008). Chalamaiah *et al.* (2009) prepared protein hydrolysate from meriga (*Cirrhinus mrigala*) egg using Alcalase and papain. DH of 62 and 17.1%

was obtained when Alcalase and papain were used for digestion at 50–55 and 60-65 C, respectively, for 90 min. The protein content of Alcalase-produced hydrolysate was higher (85%) than that of papain hydrolysate (70%). Hydrolysis by both enzymes increased protein solubility of fish egg protein hydrolysates to above 72.4% over a wide pH range (2-12). Alcalase hydrolysis of the roe protein from snakehead murrel (28.41%DH) could gain protein solubility exceeding 80% over a wide pH range (2-12). While Alcalase-hydrolyzed roe protein of rohu (18.85%DH) showed the minimum solubility at pH 2 and became gradually increase upto 50%, when pH reached to 12. This high solubility of hydrolysates was due to the generation of low molecular weight peptides by hydrolysis, which are expected to have proportionally more polar residues than the parent proteins, with the ability to form hydrogen bonds with water and increase solubility (Gbogouri *et al.*, 2004). The balance of hydrophilic and hydrophobic forces of peptides is another crucial factor determining solubility (Gbogouri *et al.*, 2004).

B. Emulsifying properties

For emulsifying properties, proteins extracted from fish and fish byproducts generally have desirable emulsifying properties (Sathivel *et al.*, 2005). Hydrolysates are surface-active materials and promote o/w emulsion because of their hydrophilic and hydrophobic groups with their associated charges (Gbogouri *et al.*, 2004). Emulsifying properties of protein and protein hydrolysate are associated with various factors including solubility (Klompong *et al.*, 2007), molecular weight, hydrophobic-lipophilic balance (Gbogouri *et al.*, 2004) and protein concentration (Thiansilakul *et al.*, 2007b), etc. Klompong *et al*. (2007) reported that hydrolysates exhibited strong emulsifying properties at low DH (5%). The increase in DH of Flavourzyme-hydrolyzed protein and Alcalase-hydrolyzed protein resulted in lower emulsion ability index (EAI) and emulsion stability index (ESI). Functional properties of hydrolysates of cod (*Gadus morhua*) backbones with varying DH were examined (Slizyte *et al.*, 2009). Increasing hydrolysis time resulted in higher DH with smaller peptide. This led to the decrease in EAI and ESI of cod backbone hydrolysates (Slizyte *et al.*, 2009). Small peptides can migrate rapidly and adsorb at the interface, but show less efficiency in decreasing the interface tension since they cannot unfold

and reorient at the interface like large peptides to stabilize emulsions (Klompong *et al.*, 2007). Higher contents of larger molecular weight peptides or more hydrophobic peptides contribute to the stability of the emulsion (Gbogouri *et al.*, 2004; Klompong *et al.*, 2007). The peptides with low molecular weight may not be amphiphilic enough to exhibit good emulsifying properties. Thiansilakul *et al.* (2007b) determined EAI and ESI of round scad protein hydrolysate at various concentrations (0.1, 0.5, 1.0 and 3%). EAI of protein hydrolysates increased with increasing concentrations, while the decrease in ESI of hydrolysates was observed at hydrolysate concentration above 0.5%. The dependence of emulsifying activity on the concentration of protein has been explained by adsorption kinetics (Matsumura and Matsumiya, 2012). At low protein concentrations, protein adsorption at the oil–water interface is diffusion-controlled. At high protein concentration, the activation energy barrier does not allow protein migration to take place in a diffusion-dependent manner. This leads to accumulation of proteins in the aqueous phase and decreased EAI of protein hydrolysate. ESI also decreased with increasing hydrolysate concentration. The increase in protein–protein interaction resulted in a lower protein concentration at the interface (Lawal, 2004; Matsumura and Matsumiya, 2012). Thus, a thinner film stabilizing the oil droplet was postulated.

C. Foaming properties

Foaming properties are physicochemical characteristics of proteins, allowing them to form and stabilize foams. The stability of foams is a consequence of the well-ordered orientation of the molecules at the interface, where the polar head is located in the aqueous phase and the hydrophobic chain faces the apolar component (Thiansilakul *et al.*, 2007b). Thiansilakul *et al.* (2007b) measured foam expansion of round scad hydrolysate at different concentrations (0.1, 0.5, 1, 3%). Foam expansions of round scad hydrolysates increased with increasing concentration (Thiansilakul *et al.*, 2007b) due to a higher rate of diffusion (Sánchez and Patino, 2005). The different enzymes used also generated hydrolysate with different foam properties. Chalamaiah *et al.* (2009) reported that foam capacities of Alcalase and papain protein hydrolysates of meriga fish egg protein were 70 and 25%, respectively. Poor foaming properties could be attributed to the small size of peptide. Souissi *et al.* (2007) produced fish

protein hydrolysate from head and viscera of Sardinella (*Sardinella aurita*) using Alcalase. The Sardinella hydrolysates at different DHs were tested for foam properties, compared to non-hydrolyzed protein. The highest foam expansion was obtained at 6.62% DH which was better than the undigested protein. However, foam formation decreased with increasing DH and also with increasing pH values. It can be explained by the small size of peptides, which would hinder the formation of a stable film around the gas bubbles and also by the presence of hydrophilic peptides during extensive hydrolysis (Souissi *et al.*, 2007). Foam formation is governed by three factors, including transportation, penetration and reorganization of molecules at the air–water interface. To exhibit good foaming, a protein must be capable of migrating rapidly to the air–water interface, unfolding and rearranging at the interface Foam stability depends principally on the nature of the film and reflects the extent of protein–protein interaction within the matrix (Thiansilakul *et al.*, 2007b). The decreased foam stability at very acidic or alkaline pH might be due to the repulsion of peptides via ionic repulsion. In general, an increase in the foam stability with increasing protein concentration has been reported as a result of the formation of stiffer foams (Lawal, 2004).

D. Fat absorption

Fat absorption capacity is an important functional characteristic, especially expected by the meat and confectionery industry (Gbogouri *et al.*, 2004). Fat binding capacity of hydrolysate is mechanically physical entrapment and depends on bulk density of protein (Yin *et al.*, 2011). Higher hydrolysis time and DH decrease fat absorption capacity of the resulting hydrolysates (Gbogouri *et al.*, 2004; Sathivel *et al.*, 2005). Sathivel *et al.* (2005) determined the effect of different enzymes (Alcalase, Flavourzyme, palatase, protex, gc and neutrase) and hydrolysis times (25, 50 and 75 min) on fat absorption capacity of red salmon hydrolysates. Among enzyme used, Flavourzyme provided hydrolysate with the best fat absorption capacity especially at minimum hydrolysis time (7.4 mL of oil/g protein), while fat adsorption capacity of catfish roe protein was 5.7 (mL of oil/g of protein) (Sathivel *et al.*, 2004). The longer hydrolysis time decrease fat absorption capacity in all hydrolysate samples. Chalamaiah *et al.* (2009) measured fat absorption capacity of two meriga

egg hydrolysates. Papain hydrolysates had higher fat absorption capacity (1.05 g/g sample), than Alcalase hydrolysates (0.91 g/g sample) The lower in fat absorption of Alcalase hydrolysates can be attributed to the high DH by Alcalase, compared to papain (Chalamaiah *et al.*, 2009). Galla *et al* (2012) reported that oil absorption capacity of roe protein hydrolysates from snakehead murrel and rohu was 1.60 and 1.0 g/g sample, respectively. Yin *et al.* (2009) examined the use of soluble and insoluble fractions from hydrolysis of catfish skin in fat absorption capacity measurement. The different fat absorption capacity is possibly due to the physical structure of those protein fractions. Gbogouri *et al.* (2004) also reported fat absorption capacity of Atlantic salmon (*Salmo salar*) head protein hydroysates at various DHs (11.50, 12.50, 14.26 and 17.30%). While nitrogen solubility increased with increasing DH, emulsion stability and fat absorption capacity decreased (Gbogouri *et al.*, 2004). The extensive hydrolysis might break many peptide bonds, thus contributing to the decrease of the oil binding properties (Souissi *et al.*, 2007).

Therefore, enzymatic hydrolysis can improve functional properties, such as emulsifying and foaming properties of protein (Gbogouri *et al.*, 2004; Giménez *et al.*, 2009; Klompong *et al.*, 2007). However, a very high degree of hydrolysis can have enormously negative effects on the functional properties (Klompong *et al.*, 2007). The solubility of protein generally increases with increasing DH, whereas greater fat absorption capacity, emulsifying and foaming properties are noticeable when DH was low (Gbogouri *et al.*, 2004).

1.2.3 Lipid oxidation/stability

1.2.3.1 Lipid oxidation

Oxidation of lipids is a major cause of deterioration of food and food products, especially those containing high content of unsaturated fatty acids. Lipid oxidation is mainly associated with the rejection by consumers due to the off-odor and off-flavor. The direct reaction of a lipid molecule with a molecule of oxygen, termed autoxidation is a free-radical chain reaction mechanism involving initiation, propagation and termination stages (Kofakowska, 2010).

Initiation

The autoxidation of fat is initiated with the formation of free radicals. Initiation reaction take place either by the abstraction of hydrogen radical from an alyllic methylene group of an unsaturated fatty acid or by the addition of a radical to double bond. The rearrangement of the double bonds results in the formation of conjugated diene (-CH=CH-CH=CH-), showing a characteristic UV adsorption at 232-234 nm (Nakayama *et al.*, 1994). The formation of lipid radical (R·) is usually mediated by trace metals, irradiation, light or heat. Also, lipid hydroperoxide, which exists in trace quantities prior to the oxidation, break down to yield radicals. Lipid hydroperoxides are formed by various pathways including the reaction of singlet oxygen with unsaturated lipids or the lipoxygenase-catalyzed oxidation of polyunsaturated fatty acids (Kofakowska, 2010)

Propagation

In propagation reaction, free radicals are converted into other radicals. Propagation of free-radical oxidation processes occurs by chain reactions that consume oxygen and yield new free-radical species (peroxy radicals, ROO·) or by the formation of peroxides (ROOH) (Kofakowska, 2010). The product R· and ROO· can further propagate free-radical reactions.

Lipid peroxy radical (ROO·) initiate a chain reaction with other molecules, resulting in the formation of lipid hydroperoxides and lipid free radicals. This reaction, when repeated many times, produces an accumulation of hydroperoxides. The propagation reaction becomes a continuous process as long as unsaturated lipid or fatty acid molecules are available (Catalá, 2010). Lipid hydroperoxides may also be formed by the reaction of an unsaturated fatty acid such as linoleic acid with oxygen in the singlet excited state or enzymatically by the action of lipoxygenase (Miyamoto and Di Mascio, 2014). Lipid hydroperoxide, the primary products of autoxidation, are odorless and tasteless (Moore *et al.*, 2012).

Termination

A free radical is any atom with unpaired electron in the outermost shell. Free radicals are electrically neutral, and salvation effects are generally very small. Owing to the bonding-deficiency and structural unstability, radicals therefore tend to react wherever possible to restore normal bonding. When there is a reduction in the amount of unsaturated lipids (or fatty acids). Thus the termination reactions lead to interruption of the repeating sequence of propagating steps of the chain reaction (Kofakowska, 2010)..

1.2.3.2 Oxidative stability of fish bulk oil and emulsion

Fish oils contain mainly triacylglycerols (TAG) and small amounts of monodiacylglycerols (MAG), diacylglycerols (DAG) and minor amounts of other non-triacylglycerol substances (Breivik and Dahl, 1992). Some of these minor substances may impact on the flavor and odor quality of the oil and affect the stability of the oil as well as its safety. Undesirable substances include moisture, insoluble impurities, free fatty acids, trace metals, oxidation products, sulfur, halogen and nitrogen compounds, pigments, sterols and organic contaminants from the environment such as polychlorinated biphenyls (PCBs) and dioxins. Genarally, oxidation of fish oils render oxidative volatiles including 1-penten-3-one (pungent green odor), *Z*-4-heptenal (fishy odor), (*E*,*E*)-2,4-heptadienal and (*E*,*Z*)-2,6-nonadienal (cucumber odor), which have been characterized as very potent odorants, contributing to the unpleasant rancid and fishy off-flavor, respectively (Iglesias *et al.*, 2007). While propanal results from oxidation of n-3 fatty acids, pentane and hexanal indicate the oxidation of n-6 fatty acids (Iglesias *et al.*, 2007). The oxidation in several fish oils and fish oil derived products revealed the formation of 1-penten-3-ol, *E*-2-hexenal, *E*-2-octenal or 1,5-octadien-3-ol by means of enzymatic mechanisms, whereas (*E*,*E*)-2,4-heptadienal, (*E*,*Z*)- 2,4-heptadienal or 3,5-octadien-2-one are believed to be derived from autoxidation mechanisms (Iglesias *et al.*, 2007). Refining of the oil aims at removing these substances while retaining desirable features. Remarkably, its unsaturated structure leads to easily oxidize and generate off-flavors.

Crude fish oil commonly contains some natural antioxidants. The most common is vitamin E or tocopherol. Fish oil and fish liver oil have different amounts of tocopherol. During processing, some of the natural antioxidants are removed. Oxidative stability was considerably lower in the fully-processed oil, compared to the crude oil. Traces of metal ions such as copper and iron are strongly pro-oxidant. They are partially removed during bleaching. Ogwok *et al.* (2008) extracted oil from belly flaps of Nile perch (*Lates niloticus*). The oil extract contained high amount palmitic, palmitoleic, stearic, oleic and docosahexaenoic fatty acids with of high vitamin A content (3.94-5.90 mg/100 g of oil). The oil extracted from Nile perch showed exceptional resistance to accelerated condition (65 °C) during storage. This might be due to its high content of *β*-carotene (2.93-4.69 mg/100 g of oil) and *α*-tocopherol (2.11-11.4 mg/100 g of oil). Frankel *et al.* (2002) reported that fish and algal oils are much more stable to oxidation in bulk systems than in o/w emulsions. The relative high oxidative stability of as algal oil (42% DHA) totally decreased after the removal of natural antioxidants by chromatographic purification. Additionally, the use of EDTA at higher concentration could inhibit oxidatioin of foods due to chelation of transitional metals as Fe, Mg, etc (Frankel *et al.*, 2002). Antioxidants are always added to prevent oxidation for retaining the taste and smell of the oil as good as possible. Natural antioxidants are preferred. Blends of *α*-, *β*-, *γ*- and *δ*-tocopherols are most commonly used. Other natural antioxidants used include flavonoids, tocopherols and carotenoids (Gan *et al.*, 2011), carnosic acid, rosemary (Wang *et al.*, 2011a), extract of garden strawberry leaf (Raudoniūtė *et al.*, 2011), etc. Some of antioxidants exhibited synergistic effects to keep down undesirable odor and taste resulting in reduction the level of antioxidants added (Frankel *et al.*, 2002; Judde *et al.*, 2003). Synthetic antioxidants as butylated hydroxyanisole (BHA), butylated hydroxytoluene (BHT), tertbutylhydroquinone TBHQ and propyl gallate have synergistic effect and much more effective than natural antioxidants (Frankel, 1998). Unfortunately, the use of synthetic antioxidants is limited as TBHQ that is not approved for use in Europe and be confined to the USA. Therefore, the uses of synthetic antioxidants are in decline due to the perception that natural food ingredients are better and safer than synthetic. Judde *et al*. (2003). reported the synergistic effect of 1% lecithin and γ-, α-tocopherol. High proportion of phosphatidylcholine and phosphatidylethanolamine in

the presence of γ-, α-tocopherol could retard the oxidation effectively. The main antioxidant mechanism of lecithins was due to a synergistic effect between amino-alcohol phospholipids and γ- and α-tocopherols (Judde *et al.*, 2003).

1.2.4 Use of emulsifier and antioxidant in food systems

Deterioration of food because of lipid oxidation results in physical and chemical change, affects consumer acceptance, human health and product cost. Lipid in food system is most likely dispersed in water as o/w emulsion. Since the higher total surface area of lipid droplets in emulsion is observed, emulsion is extremely sensitive to oxidation, compared to bulk oils. To retard lipid oxidation, antioxidants are generally applied in food emulsions. The effectiveness of antioxidants in food emulsions depends on both their chemical and physical properties. Polar antioxidants are preferred in oil-in-water emulsions, while nonaupolar antioxidants are more effective in bulk oils (Yuji *et al.*, 2007). The antioxidative activity in o/w emulsions could also be dependent on their surface activity and ability to accumulate at the oil-water interface, where oxidative reactions are most prevalent (Yuji *et al.*, 2007).

Nowsaday, the enrichment of n-3 PUFA in food products such as meat products, milk products and bread and bakery products has been widely studied (Kolanowski and Laufenberg, 2006; Nordvi *et al.*, 2007a; Pelser *et al.*, 2007). The consumption of n-3 PUFA enriched food product is suggested to enhance potential health benefits, especially in protection against cardiovascular diseases (CVD), cancer and improvement of brain development and function (Kolanowski and Laufenberg, 2006). Since high susceptibility towards oxidation of fish oil, enriched food products should be stored not over long periods of time, unless under special storage conditions and in a special type of packing, especially avoiding an exposure to air. Otherwise the desirable n-3 PUFA may easily transform into toxic peroxides and other by-products of lipid oxidation promoting cancer development (Kolanowski and Laufenberg, 2006). Pelser *et al.* (2007) reported that the substitution of 10 and 15% pork fat using encapsulated fish oil in production of ductch style sausage could be accomplished with acceptable physical and sensory analysis. The addition of fish oil rich in DHA in pre-emulsified form were also accomplished in Spanish bologna-type sausage

(Cáceres *et al.*, 2008). Quality of sausage as a food emulsion product depends on lipid and protein stability. The oxidation of lipid generated free radicals which is believed to promote the oxidative damage of proteins via the prooxidant activity of primary (hydroperoxides) and secondary (aldehydes, ketones) lipid oxidation products. Protein oxidation mainly occurs via free radical reactions in which peroxyl radicals are generated in the first stages of PUFA oxidation can abstract hydrogen atoms from protein molecules, leading to the formation of protein radicals (Cabiscol *et al.*, 2010).The formation of non-covalent complexes between lipid oxidation products and reactive amino acids residues, as well as the presence of some particular metal, such as copper and iron, can also lead to protein radical generation (Cabiscol *et al.*, 2010; Lund *et al.*, 2011). The influence of protein oxidation on the certain quality associated with pigment degradation, color and texture changes has been reported (Estévez and Cava, 2004; Estévez *et al.*, 2007).

In order to inhibit the development of oxidative reactions in meat products, natural or synthetic antioxidants have been commonly used in the meat industry (Estévez and Cava, 2006). USDA regulations (USDA, 2005) permit up to 0.01% (based on fat content) each of BHA and BHT in precooked bacon and up to 0.002% (based on total essential of the spice mixture) each in sausage. Estévez *et al.* (2007) studied the oxidative stability of frankfurters during storage at 4 °C for 60 days. The refrigerated frankfurter containing high amount of vitamin E and total phenolic contents exhibited the highest oxidative stability. The addition of phenolic compounds as rosemary essential oil at different contents (150, 300 and 600 ppm) on lipid and protein oxidation of frankfurters during storage at 4 °C was studied (Estévez and Cava, 2006). The rosemary essential oil at 150 ppm could successfully inhibited the oxidation development of lipid and protein (Estévez and Cava, 2006). Efficient combinations of antioxidants for the stabilization of bulk fish lipid high in γ- or δ-tocopherol and low α-tocopherol in combination with lecithin or citric acid esters from monoglycerides (Citrem) and ascorbyl palmitate have been reported (Drusch *et al.*, 2008). Recently, it has been reported that protein and protein hydrolysate could play emulsifier properties concomitant with antioxdative activity (Faraji *et al.*, 2004; Nieto *et al.*, 2009). Faraji *et al.* (2004) studied the prevention of oxidative

deterioration by using whey protein isolate (WPI), soy protein isolate (SPI) and sodium caseinate (CAS) as emulsifier antioxidants. The oxidative stability of emulsions containing different kinds of protein in the continuous phase decreased in the order SPI > CAS > WPI, as determined by both hydroperoxide and headspace propanal formation. Iron-binding studies showed that the chelating ability of the proteins decreased in the order CAS > SPI > WPI. The free sulfhydryls of both WPI and SPI were involved in their antioxidant activity. Tong *et al.* (2000) reported that the ability of continuous phase whey proteins to inhibit lipid oxidation was due to a combination of free radical scavenging by free sulfhydryl groups and chelation of prooxidant metals. Casein and its derivative can inhibit lipid oxidation by scavenging the free radical intermediates and chelating prooxidant metals (Ries *et al.*, 2010). Alcalase-hydrolyzed potato peptides have been reported as emulsifier antioxidant in cooked frankfurters (Nieto *et al.*, 2009). The addition of Alcalase-hydrolyzed potato peptide about 2.5% resulted in the reduciton of cooking losses and fracture force, and had a significant inhibitory effect on lipid oxidation in cooked frankfurters (Nieto *et al.*, 2009). Alcalase-hydrolyzed potato peptide could also function as emulsifier and antioxidant in soybean o/w emulsion (Cheng *et al.*, 2010). The incorporation of tween 20 and Alcalase-hydrolyzed potato peptide could reduce formation of peroxides up to 53.4% and malonaldehyde-equivalent substances up to 70.8% after 7 days of storage at 37 °C, when compared with potato protein hydrolysate-free emulsions (Cheng *et al.*, 2010).

1.2.5 Phenolic compounds

Phenolic compounds have gained increasing interest as food additives and functional foods. Numerous investigations have shown that phenols exhibit potentially positive effects including anti-inflammatory (Mamani-Matsuda *et al.*, 2006), anti-carcinogenic and anti-microbial activities (Jakobek *et al.*, 2009; Puupponen-Pimiä *et al.*, 2005). The beneficial effects of phenols have also been ascribed to their strong antioxidant activity, mainly associated with their ability to scavenge oxygen radicals and other reactive species (Jakobek *et al.*, 2009). Radical scavenging activity of phenolic compounds depends on the number and position of hydroxyl (–OH) groups, methoxy (- OCH_3) substituents and glycosylation in the

molecules (Cai *et al.*, 2006). Additionally, the substitution patterns in the B-ring and A-ring as well as the 2,3 double bond (unsaturation) and the 4-oxo group in the C-ring also affect antioxidant activity of phenolic (Mustafa *et al.*, 2010). For flavonoids, the positions of 3′, 4′-orthodihydroxy configuration in ring B and 4–carbonyl group in ring C were responsible for electron donating properties (Mustafa *et al.*, 2010). Cai *et al.* (2004) evaluated 112 anticancers from traditional Chinese medicinal plants. The majority phenolic compounds of 112 herbs were identified as phenolic acids, flavonoids, tannins, coumarins, lignans, quinones, stilbenes and curcuminoids. Mustafa *et al.* (2010) demonstrated that 21 tropical plant extracts possessed DPPH radical scavenging activity. Among those tropical plants, *Cosmos caudatus* (IC_{50} = 21.3 μg/mL) and *Piper betle* (IC_{50} = 23.0 μg/mL) extracts, had antioxidant activity comparable to α-tocopherol and BHA. Apart from radical scavenging activity, some phenolics are also able to quench a variety of transitional metals (pro-oxidants), which are available in food systems (Ozsoy *et al.*, 2008). The polyphenols possessing various bioactivities were presented in Table 4. Polyphenol extracted from various plants also exhibited antimicrobial effects (Cho *et al.*, 2008; Daglia, 2012). Korean green tea extract containing five main polyphenolic compounds (EGCG, EGC, gallocatechin gallate, epicatechin and ECG), had antibacterial effect against 13 strains of methicillin-resistant *Staphylococcus aureus* (MRSA) clinical isolates and 17 strains of methicillin-susceptible *Staphylococcus aureus* (MSSA) (Cho *et al.*, 2008). The antibacterial activity of polyphenols is posibly attributed by interaction with microbial membrane proteins, thereby hindering the adherence of bacterial cells (*Streptococcus mutans*) to the tooth surface (Ferrazzano *et al.*, 2011). Rukayadi *et al.* (2008) reported anti-biofilm activity of macelignan, isolated by nutmeg (*Myrisica fragrans* Houtt.) against oral bacteria including *S. mutans, S. sanguinis* and *Actinomyces viscosus*. Skin extract of white grape cultivars containing plenty of phenolic compounds exhibited activity against food borne pathogens including *Campylobacter* and *Salmonella* (Katalinić *et al.*, 2010). Apart from their bioactivities, phenolic compounds can be used to modify protein functionality (Aewsiri *et al.*, 2013; Balange and Benjakul, 2009).

Table 4 Bioactivities of the phenolic compounds extracted from varying plants

Plant	Major polyphenol	Bioactivity	Reference
Trumpet vine (*Campsis radicans* (L.) flower	Quercetin, apigenin, cyandin-3-rutoside and ferulic acid	Antioxidant (ABTS radical scavenging activity)	(Cai *et al.*, 2004)
Hot pepper (*Capsicum annuum* L.) fruits	Quercetin, ferulic acid and capsaicin	Antioxidant (β-carotene–linoleic acid system and DPPH radical scavenging activity)	(Materska and Perucka, 2005)
Oregano (*Origanum vulgare)*	Quercetin, apigenin and myricetin	Antioxidant (β-carotene–linoleic acid system)	(Škerget *et al.*, 2005)
Hawthorn (*Crataegus laevigata*)	Quercetin and apigenin	Antioxidant (β-carotene–linoleic acid system)	(Škerget *et al.*, 2005)
Smilax excelsa L. leaves	Phenolic acids and flavonoids	Antioxidant (Hydroxyl and hydrogen peroxide scavenging activity, chelating activity, linoleic acid system and β-carotene bleaching system)	(Ozsoy *et al.*, 2008)
Sonchus asper	Carotenoids, catechin, rutin, quercetin, etc.	Antibacterial, antitumor and antioxidant (DPPH, ABTS, hydroxyl and superoxide anion radical scavenging activities and chelating activity)	(Khan *et al.*, 2012)
Solidago microglossa leaves	Quercetin and Gallic acid	Antioxidant (DPPH and hydroxyl radical scavenging activites)	(Sabir *et al.*, 2012)

1.2.5.1 Protein interactions with phenolic compound

Polyphenols can interact with proteins, leading to the inhibition of the enzymes, decrease of the protein digestibility and the induction of protein precipitation. Such an interaction directly affect protein functionality (Rawel *et al.*, 2001; Sarker *et al.*, 1995). Polyphenols can interact with proteins in two different ways: via non-covalent (reversible) interactions and via covalent interactions, which in most cases are irreversible (Balange and Benjakul, 2009). Complexation between polyphenols and proteins leads to different solubility behavior (Balange and Benjakul, 2009; Luck *et al.*, 1994). The interaction may occur via multisite interaction (several phenolic compounds bound to one protein molecule) or multidentate interaction (one phenolic compound bound to several protein sites or molecules). The type of interaction depends on the type and the molar ratio of both phenolic compound and protein (Kroll *et al.*, 2003; Prigent *et al.*, 2008).

Interaction between proteins and polyphenols can be influenced by various factors, such as the solution conditions (solvent composition, ionic strength and pH), temperature, time (Da Silva *et al.*, 1991; De Freitas and Mateus, 2001; Kawamoto and Nakatsubo, 1997; Prigent *et al.*, 2003) and type of polyphenol (Kroll *et al.*, 2003; Rawel *et al.*, 2001). In addition, the degree of the polymerization and esterification may affect the binding affinities (Bacon and Rhodes, 1998). Phenolic hydroxyl groups on both A- and B-rings in the polyphenol molecule play an important role in the protein-phenol complex formation (Kawamoto *et al.*, 1996; Simon *et al.*, 2003).

The stability of the polyphenol-protein complexes are governed by many groups present in the amino acids (Prigent *et al.*, 2003; Vergé *et al.*, 2002). Phenolic compounds act as a polydentate ligand on the protein surface involving hydrogen bonding as well as hydrophobic effects. The aromatic groups of polyphenols are supposed to be involved in a face-to-face stacking with amino acid residues of linear proteins, whereas the interaction with globular proteins, such as α-lactalbumin and BSA, probably involves only surface exposed residues (Carvalho *et al.*, 2004). Protein structure has an influence on the formation of complexes with

polyphenols through hydrogen bonding (De Freitas and Mateus, 2001; Hagerman *et al.*, 1998; Naczk *et al.*, 2001; Siebert *et al.*, 1996). The protein-polyphenol complex formation is usually strongest just below the isoelectric point of proteins where the protein-protein electrostatic repulsion is minimized (Hagerman and Butler, 1981).

A. Non-covalent interaction between proteins and phenolic compounds

(1) Hydrogen bonding

During protein-polyphenol interaction, a strong hydrogen bond is formed between the carbonyl groups of the amino acids or peptide backbone and hydroxyl group in the polyphenol (De Freitas and Mateus, 2001; Hagerman *et al.*, 1998). Hydrogen bond is important in the complex formation (Luck *et al.*, 1994). The hydrogen bond between phenolic hydroxyl group and the peptide carbonyl groups of the protein are in aqueous media solvated with hydrogen-bonded water molecules (Kroll *et al.*, 2003). Therefore, these solvent bonds have to be broken before interaction can take place (Kroll *et al.*, 2003).

Phenolic hydroxyl groups that are located close to other hydrogen-bonding groups (1,2-dihydroxy and 1,2,3- trihydroxy groups) generally form hydrogen bonds with themselves and this reduces their capacity to form external hydrogen bonds with proteins. The binding affinity parallels with the number of 1,2-dihydroxy rings in the polyphenol molecule (Charlton *et al.*, 2002). The structure of protein has an effect on binding mechanism (Frazier *et al.*, 2003). Proline and arginine residues in proteins can form hydrogen bonds. Arginine has a long flexible hydrophobic side chain with the hydrogen-bond donating guanidino group. The enhanced ability of the proline-rich proteins to interact with the phenolic compounds is related to their flexible secondary structure and the greater extent of hydrogen bonding. Carbonyl group of tertiary amides is a better hydrogen bond acceptor than the carbonyl group of primary or secondary amides (Luck *et al.*, 1994; Murray *et al.*, 1994; O'Connell and Fox, 2001).

(2) Hydrophobic interactions

The protein-polyphenol complexes are also stabilized by hydrophobic interactions. In hydrophobic bonding, the aliphatic (basic) and aromatic side chains (hydrophobic regions) of amino acids can interact with the aromatic nuclei of the polyphenol and the complexes can be formed in which proline residues play a key role (Baxter *et al.*, 1997; Hagerman *et al.*, 1998; Lu and Bennick, 1998; Wroblewski *et al.*, 2001). The proline residues (pyrrolidine rings) are good hydrophobic binding sites since they provide an open, flat, rigid, hydrophobic surface favorable for association with other flat, hydrophobic functions, such as aromatic rings in the polyphenols (Charlton *et al.*, 2002; Luck *et al.*, 1994; Murray *et al.*, 1994). The hydrophobic interaction usually occurs between the least sterically hindered amino acids and phenolic rings available for intermolecular interaction (Murray *et al.*, 1994; Oh *et al.*, 1980). Besides proline residues, histidine, arginine, phenylalanine, tryptophan, lysine, cysteine and methionine can participate in the interaction reaction (Baxter *et al.*, 1997; Charlton *et al.*, 2002; Suryaprakash *et al.*, 2000). In histidine residues, imidazole ring interacts with aromatic components of polyphenols. Polyphenols interaction with tryptophan is most likely due to the partial positive charge present at the –NH group of the indole moiety (Suryaprakash *et al.*, 2000). Lysine, as well as arginine, has hydrophobic section in the side chain which may take part in the interaction. In addition, lysine has positive charge near neutral pH, leading to interaction with the carboxylate group or the aromatic ring of the polyphenol (Suryaprakash *et al.*, 2000). Charlton *et al.* (2002) concluded that polyphenols bind through hydrophobic interactions reversibly and relatively weakly at each individual binding site.

Precipitation of the protein-polyphenol complexes is likely to occur via interactions between exposed phenolic rings on one complex and proline or phenolic rings on the other (Charlton *et al.*, 2002). The interaction phenomenon takes place in the beginning of reactions, leading to the formation of stable complexes (Da Silva *et al.*, 1991). Initially added polyphenols bind to the protein and soluble complexes are formed. In general, several polyphenol molecules can bind to the same protein. When more polyphenol is added, until the second stage, where there is enough polyphenol

bound to the protein to act as a linker between two protein molecules. The protein then forms a polyphenol-coated dimer, which start to precipitate. In the final stage, the complex aggregates into either small particles or large particles. Larger protein molecules tend to bind polyphenol more tightly because increasing length of protein allows it to fold and "wrap around" the polyphenol molecule, form intramolecular interactions and interact at several places at the same time (Charlton *et al.*, 2002). Usually the number of amino acids blocked in the protein by phenolic compounds is higher than the amount of phenolic compound bound (Rawel *et al.*, 2002a). Therefore, some protein groups must be involved in intra- and intermolecular cross-linking through hydrophobic interactions (De Freitas and Mateus, 2001; Kroll *et al.*, 2003; Rawel *et al.*, 2002b).

B. Covalent interaction between proteins and phenolic compounds

The covalent binding of the phenolic compounds to the proteins occurs through oxidized phenolic substances (quinones). These quinones can be formed enzymatically or non-enzymatically. In food industry, quinones are acquired via the increase pH, inducing the deprotonation of the phenolic hydroxyl group (Yabuta *et al.*, 2001). Quinones can bind covalently to the proteins at nucleophilic functional groups, such as lysine, methionine, histidine, cysteine, tyrosine and tryptophan residues, limiting the digestibility of the protein molecule (Figure 2) (Carbonaro *et al.*, 1996; Hurrell *et al.*, 1982; Rawel *et al.*, 2001). The formation of the quinone-protein complexes, as well as formation of the other polyphenol-protein complexes, can lead to polymerization (Kroll *et al.*, 2003). Quinones are electrophilic and can form cross-links with either sulfhydryl or amino groups of proteins (O'Connell and Fox, 2001). The protein-quinone interaction can change the isoelectric point of protein to the lower pH values due to the introduction of carboxylic groups following the covalent attachment of the phenolic acids and by the parallel blocking of the lysine residues in protein (Rawel *et al.*, 2001; Rawel *et al.*, 2002b). The quinones can react very efficiently with lysine residues in proteins (Hurrell *et al.*, 1982; Rawel *et al.*, 2001). Phenol-protein complexes formed under oxidizing conditions are more stable than those formed in the absence of oxidants (Chen and Hagerman, 2004).

The covalent interaction between oxidized phenolic compounds and amino acid residues of the proteins allows the formed complex to act as antioxidants as long as there are free phenolic hydroxyl groups in one of the phenolic rings (Almajano and Gordon, 2004; Arts *et al.*, 2002; Rohn *et al.*, 2004). However, the antioxidant activity of the quinone-protein complexes is less than the antioxidant activity of free phenolic compounds (Rohn *et al.*, 2004). The degree of masking of antioxidant capacity depends on both the type of polyphenol and the type of protein (Arts *et al.*, 2002). The cross-linking and polymerization is partly responsible for the loss of the antioxidative ability of covalently bound phenolic compounds. With an increasing amount of proteins present, the reactive sites of phenolic compound are more involved in the protein-phenolic-protein reactions (Rohn *et al.*, 2004). Additionally, steric hindrance could also be responsible for the decreased activity.

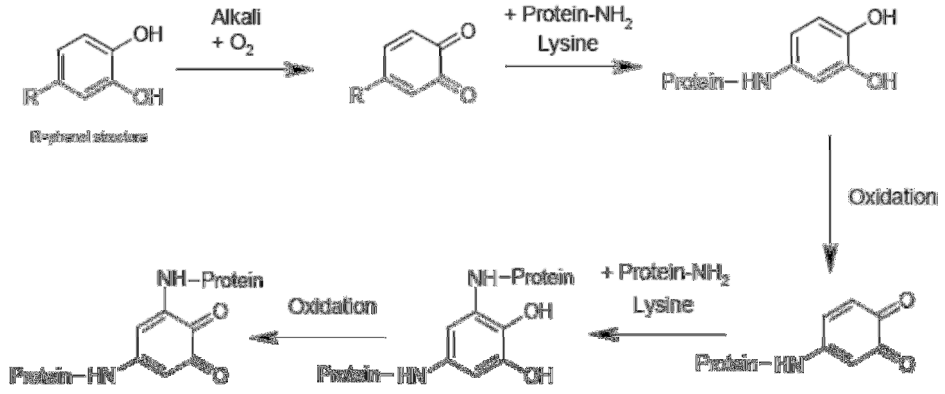


Figure 2 Interaction reaction between lysine side chain of protein and quinone form of polyphenol, leading to polymerization and complex formation

Source: Rawel *et al.* (2001)

1.2.5.2 Tannic acid and oxidized tannic acid

Tannic acid, a naturally occurring plant polyphenol, is composed of a central glucose molecule derivatized at its hydroxyl groups with one or more galloyl residues (Gülçin *et al.*, 2010). The polyphenolic nature of tannic acid consisting hydrophobic core and hydrophilic shell is the features responsible for its antioxidant action (Isenburg *et al.*, 2006). As a food additive, safe dosage of tannic acid ranges

from 10 to 400 μg, depending on the type of food to which it is added (Chen and Chung, 2000). For polyphenol-protein interaction aspect, tannin which contains sufficient hydroxyls and other groups such as carboxyls has been found to form strong complexes with the proteins and other macromolecules (Kroll *et al.*, 2003). The incorporation of oxidized tannic acid into myofibrillar protein of bigeye snapper (*Priacanthus tayenus*) rendered the film with higher mechanical properties (stronger and stiffer film structure) (Prodpran *et al.*, 2012). The addition of oxidized tannic acid (OTA) resulted in increase in gel strength of mackerel and bigeye snapper surimi (Balange and Benjakul, 2009). Balange and Benjakul (2009) suggested that the increase in gel strength might be attributed to hydrophobic interactions between phenolic compounds and hydrophobic amino acids such as alanine, valine, isoleucine, leucine, methionine, phenylalanine, tyrosine, tryptophan, cysteine and glycine residues (Prigent, 2005). Aewsiri *et al.* (2009) reported that modification of gelatin with oxidized tannic acid (OTA) could enhance antioxidative activity but had negative effect on its emulsifying property.

1.2.6 Encapsulation of fish oil

Recently, the microencapsulation technique has been used to apply for prolong shelf life of the fish oil emulsion (Drusch *et al.*, 2007); Shen *et al.*, 2010). Microencapaulation is believed to protect fish oil against oxidation by limiting undesirable influences of the environment, i.e., oxygen, light, humidity, etc. (Aghbashlo *et al.*, 2013a). The use of fish oil in dry encapsulated form also enables to incorporate n-3 PUFAs in instant foods, infant formula and bread. Biopolymer composites and coacervates have been found to be useful for the formulation of microencapuslated oils, thereby stabilizing oils (Drusch and Mannino, 2009; Serfert *et al.*, 2009). Combination of protein with other materials (sugars or polysaccharides; maltodextrin (MD); starch and nonstarch polysaccharides such as gums, celluloses and chitosan; and modified starch alone or in combination with gum Arabic) have been successful to stabilize PUFAs rich oil emulsion (Drusch *et al.*, 2007).

1.2.6.1 Emulsion

Emulsion is defined as a system, which consists of two immiscible liquids (usually oil and water). One of the liquids disperse as small spherical droplets (*dispersed phase*) in the other (*continuous phase*). Based on stability, emulsion can be categorized into three systems including coarse emulsion, microemulsion and nanoemulsion (Burguera and Burguera, 2012). Coarse and nano-emulsions are kinetically stable, whereas microemulsions are thermodynamically stable (Burguera and Burguera, 2012). Coarse emulsion has a cloudy appearance. The droplet size is typically between 0.5 and 50 μm. Metastable systems of coarse emulsion lead to destabilization via several mechanisms (creaming, coalescence, flocculation, etc.) (Bengoechea *et al.*, 2010). The appearance of microemulsions is optically transparent. For a mini- or nanoemulsion, the appearance may be translucent (0.05–0.2 μm) or turbid (upto 0.5 μm) upon the droplet size and the refractive index difference between the droplets and the continuous phase (Burguera and Burguera, 2012). The use of emulsifiers such as proteins or surfactants is required to stabilize emulsion. The process of converting two separate immiscible liquids into an emulsion, or of reducing the size of the droplets in a preexisting emulsion, is known as *homogenization*. In the food industry, this process is usually carried out using mechanical devices known as *homogenizers*, which usually subject the liquids to intense mechanical agitation using high speed blenders, high-pressure valve homogenizers and colloid mills (McClements, 2005).

A High pressure homogenizer

To obtain emulsion with stability to gravitational separation, mechanical devices such as high-pressure valve homogenizers, microfluidizers, etc. are used. This equipment can generate intense disruptive forces that breakup the oil and water phases and lead to the formation of tiny oil droplets (Qian and McClements, 2011). Diagram of high pressure homogenizer is illustrated in Figure 3.

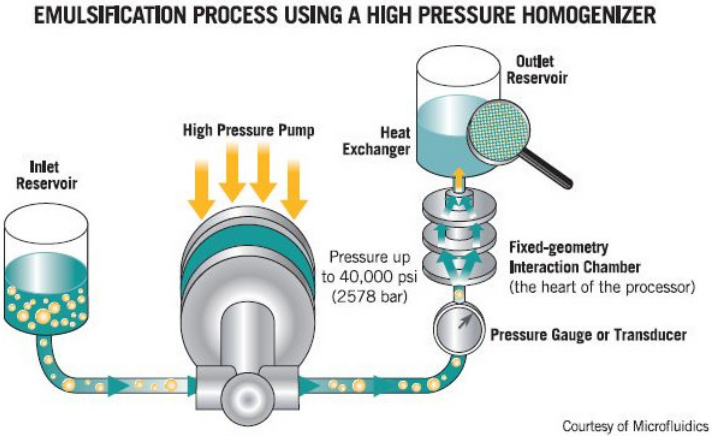


Figure 3 Schematic diagram of Microfluidizer

Source: Particle Sciences Drug and Development Service (2009)

High pressure homogenization mechanically reduces the size of globules to less than 1 μm and is used to produce emulsion of uniform composition and higher stability (Lee *et al.*, 2009). The mean particle diameter of the emulsion decreased with increasing homogenization pressure and number of passes. When particle size was decreased, the attractive forces acting between the droplets were reduced. Thus the better emulsion stability against droplet flocculation and coalescence can be achieved (Qian and McClements, 2011).

B Factor influencing emulsion stability

(1) Type and concentration of emulsifier/surfactant

The type and concentration of emulsifier present prior to homogenization clearly had a major impact on the size of the droplets produced (Qian and McClements, 2011). When comparing between small-molecule surfactants (like SDS and Tween) and biopolymer, it was found that small molecule surfactants are more effective at making small droplets than biopolymers (caseinate and β-lactoglobulin) under similar homogenization conditions because they adsorb to the droplet surfaces more rapidly (Qian and McClements, 2011) At sufficiently low concentrations, surfactants or emulsifiers exist as monomers in solution because the entropy of mixing overweighs the attractive forces operating between the molecules (Jönsson *et al.*, 1998). Nevertheless, as their concentration is increased they can

spontaneously aggregate into a variety of thermodynamically stable structures known as ‘association colloids’, for example, micelles, bilayers, vesicles and reverse micelles (McClements, 2005)

(2) pH and ionic strength

In general, protein-stabilized emulsions are particularly sensitive to pH and ionic strength effects. They tend to flocculate at pH values close to the isoelectric point of the adsorbed proteins and when the ionic strength exceeds a certain level (McClements, 2004). For sodium caseinate stabilized emulsion, gradual acidification of the emulsion towards the protein isoelectric point (pI ≈ 4.6) increases the net attractive forces between the casein molecules, resulting in a self-association of the adsorbed and non-adsorbed protein components (Allen *et al.*, 2006). This could lead to emulsion destabilization.

(3) Temperature

Emulsions stabilized by globular proteins are also particularly sensitive to thermal treatments, because these proteins unfold when the temperature exceeds a critical value exposing reactive nonpolar and sulfhydryl groups. These reactive groups increase the attractive interactions between droplets, which may lead to droplet flocculation (McClements, 2005).

1.2.6.2 Wall material

The physicochemical properties of the wall material are critical factor in protecting and/or controlling the release of core materials (Drusch *et al.*, 2007). Their physicochemical properties including solubility, molecular weight, glass, melting transition, crystalline, diffusibility, film forming and emulsifying properties affect the encapsulation efficiency. Polymer substances as carbohydrate, protein and gum were used as a wall material. The use of improper polymer as low molecular weight carbohydrates may promote caking, collapse and re-crystallization of wall material during storage (Partanen *et al.*, 2002). On the other hand, the use of low-molecular-weight carbohydrates such as sucrose and glucose individually has been

reported for spray drying problems due to their caramelization properties, adherence to the surface of the spray-dryer and heterogeneous form that caused the clogging of the nozzle (Gharsallaoui *et al.*, 2012).

Owing to excellent interfacial properties, protein based microencapsulation is considerable attention (Wang *et al.*, 2011b). Amphiphilic characteristic, molecular conformation and high diffusivity allow the well-defined distribution around the fat globule surface can be sustained from protein. In addition, proteins possess high binding properties for the flavor compounds (Gharsallaoui *et al.*, 2007). The most commonly used proteins for encapsulating food ingredients by spray-drying are milk proteins (sodium caseinate and whey protein) and gelatin. Upon spray drying, the adsorption process of proteins at the air–water interface contains three steps; (i) diffusion of molecules from the bulk solution to the subsurface region; (ii) the adsorption of molecules from a subsurface to the air-water interface; and (iii) the reconformation or rearrangement of adsorbed molecules within the surface layer (Jayasundera *et al.*, 2009). The smaller the size of the proteins, faster the adsorption of proteins to the air-water interfaces. Charve and Reineccius (2009) studied the flavor encapsulation prepared by three proteins (sodium caseinate, whey or soy protein isolates). Whey protein isolate gave the highest flavor retention (87%).This might be due to whey protein localized at the interface create a more elastic interface compared to casein (Jayasundera *et al.*, 2009). However, protein denaturation, especially globular proteins, can occur at condition, by which high temperature and high water activity of the drying droplet presented together. Millqvist-Fureby *et al.*(2001) reported that spray-drying resulted in a denaturation and aggregation of β-lactoglobulin. This drying process also affected the functional properties of spray-dried powder prepared by whey proteins (Millqvist-Fureby *et al.*, 2001). During drying process, protein film at the interface of the emulsion, which is completely hydrated and the loss of water would result in the shrinkage of the film. Jayasundera *et al.* (2009) found that the combination of sodium caseinate with lactose could reduce the shrinkage of microcapsule. This might be due to the lactose can replace the water to some extent and keep the protein solubilized after drying and thereby lactose reduces the shrinkage. This leads to the increase in stability of the sodium caseinate

film on the powder surface, and less fat leaks out onto the powder surface (Jayasundera *et al.*, 2009). Pourashouri *et al.* (2013) investigated cod oil stability as affected by varying wall materials including fish gelatin without and with-combination of chitosan, maltodextrin and/or MTGase. Among all wall materials, gelatin-MTGase-maltodextrin had the highest encapsulation efficiency (88%) with decreasing amount of surface oil (2.63%). Pourashouri *et al.* (2013) suggested that the reaction between the amino groups of gelatin and the carbonyl group of maltodextrin (Maillard reaction) might have a significant contribution to the formation of the tough skin crust, leading to reducing the diffusion of the entrapped oil to the surface of the particles. Additionally, MTGase might facilitate covalent cross-link formation, producing a compact film with improved encapsulation efficiency (Pourashouri *et al.*, 2013).

Ratio of core to wall material also affects with the microencapsulated oil quality. Aghbashlo *et al.*, (2012) studied the effect of oil concentration (10-30%, based on total solids) and water content (70-90%) on encapsulation efficiency when skim milk protein was used as wall material for fish oil microcapsulation. Aghbashlo *et al.* (2012) found that increasing oil proportion in total solids negatively affected the encapsulation efficiency at the same water content. This was attributed to insufficient amount of wall material. As a consequence, wall material could not completely cover the oil droplets at the higher oil proportion (Jafari *et al.*, 2008b; Zhong *et al.*, 2009).

1.2.6.3 Preparation of infeed emulsion

The formation of surface indentations in spray dried powders is associated with composition of wall materials, drying parameters and emulsification method used to prepare infeed emulsions (Jafari *et al.*, 2008a). Infeed emulsion with appropriate droplet size and viscosity has significant effect on the encapsulated product. The size of atomized droplet is directly proportional to the viscosity of feed at constant atomizing condition (Aghbashlo *et al.*, 2012) and affects lipid losses during atomization shearing (Gharsallaoui *et al.*, 2012). Excessive viscosity adversely affects to atomization process and drying rate, leading to generation of elongated and large droplets. Generally, conventional emulsion suffer from physical instability when

exposed to environmental stresses such as heating, chilling, freezing, drying, pH and ionic strength (Gharsallaoui *et al.*, 2012). Homogeneous and fine oil-in-water emulsions, is necessary for microencapsulation by spray drying (Gharsallaoui *et al.*, 2012). Jafari *et al.*(2008a) studied characteristics of the spray dried powders prepared by whey protein emulsions with three different emulsification methods including Silverson (rotor-stator system), ultrasound and Microfluidizer. Powders produced from Microfluidized emulsions provided the minimum surface oil coverage (18%), followed by ultrasound (20%) and Silverson (rotor-stator system) (35%), respectively. The morphology of the obtained powers was varied. Ramakrishnan *et al.* (2013b) examined emulsion stability of the fish oil emulsions as affected by rotor stator homogenization and premix membrane emulsification (nylon and nitrocellulose mixed ester). The use of rotor stator for emulsification could provide emulsion with polydisperse oil droplet. On the other hand, emulsion obtained from premix membrane emulsification (ME) was monodisperse with particle size range of 1-2 µm, regardless of membrane types. After spray drying, powder of ME-emulsion had smaller particle size (9.12 µm), less amount of surface oil (0.084 g/g powder) and propanal content (70 mg/kg), compared to those prepared by rotor stator (particle size = 13.31 µm; surface oil content = 0.117 g/g powder; propanal content = 120 mg/kg).

Microencapsulation efficiency can be increased by increasing wall solution solid concentration. This can be related to the effect of wall solid concentration on the formation of surface core prior to the formation of crust around the drying droplets (Young *et al.*, 1993). Kha *et al.* (2010) found that a good quality Gac powder in terms of color, total carotenoid content and total antioxidant activity can be produced by spray-drying at inlet temperature of 120 ºC in the presence of maltodextrin (10%, w/v). The main limitation of the spray-drying technique in microencapsulation is the limited number of wall materials available and that must have a good solubility in water (Fuchs *et al.*, 2006). Barley protein-stabilized fish lipid microcapsules (1–5 µm) were successfully prepared by a pre-emulsifying process. The optimal conditions for microcapsule formation were 15% protein and a 1.0 oil/protein ratio. Microcapsules could be converted into free-flowing powders by a spray-drying process at an optimum inlet temperature of 150 °C. These microcapsules

exhibited high lipid encapsulation efficiency, loading efficiencyand low moisture content (Wang *et al.*, 2011b).

1.2.6.4 Drying

Several microencapsulation approaches are applied such as spray-drying, spray-cooling, spray-chilling, air-suspension coating, extrusion, freeze-drying, coacervation, co-crystallization, liposome entrapment, molecular inclusion, etc. (Gharsallaoui *et al.*, 2007). Comparing to the others methods, microencapsulated fish oil produced by freezing and then lyophilization yielded high quality and stability. Practically, spray drying as a dehydration process is a popularly used in food industry due to low cost and available equipment. The disadvantage of spray drying technique is high temperature during process may accelerate the oxidation process, the nutrition loss may appear (Klaypradit and Huang, 2008). Additionally, further step such an agglomeration process is required to produce finely microencapsulated products. A large variety of morphological microcapsules including simple, multi-core, multi-wall, matrix and irregular were generated during encapsulation process (Estevinho *et al.*, 2013). The characteristic of the microencapsulated products involves in several factors such as type of wall material, proportional of wall and core materials and encapsulation technique used, etc (Estevinho *et al.*, 2013).

A. Feed temperature and feed rate

In fact, feed temperature affects the emulsion properties such as viscosity, fluidity and capacity to be homogeneously sprayed (Gharsallaoui *et al.*, 2007). When the feed temperature is increased, viscosity and droplets size decrease. However, very high temperatures can cause volatilization or degradation of some heat-sensitive ingredients. The rate of feed delivered to the atomizer is adjusted to ensure that each sprayed droplet reaches the desired drying level before it comes in contact with the surface of the drying chamber.

B Air inlet temperature

Air inlet temperature is directly proportional to the microcapsule drying rate and the final water content. When the air inlet temperature is low, the low evaporation rate causes the formation of microcapsules with high density membranes, high water content, poor fluidity and easiness of agglomeration. However, a high air inlet temperature causes an excessive evaporation and results in cracks in the membrane inducing subsequent premature release and a degradation of encapsulated ingredient or also a loss of volatiles. In case of a wall material having strong film forming properties, the use of high inlet and outlet air temperature may lead to ballooning (Drusch and Berg, 2008). Ballooning is a phenomenon, which is caused by fast fixing of the particle structure in the early stage of drying with subsequent steam formation in the interior of the particle and inflation of the particle (Drusch *et al.*, 2007). Drusch *et al.*(2007) reported that ballooning of fish oil microcapsules prepared using nOSA-starch type 1 occurred, when drying temperature of 210/90 °C was conducted. The air inlet temperature is usually determined by two factors: the temperature which can safely be used without damaging the product or creating operating hazards. The comparative cost of heat sources must be considered (Zakarian and King, 1982). Chen *et al.* (2013) studied the effect of air inlet temperatures (160, 170 and 180 °C) and air outlet temperatures (70 and 80 °C) on the characteristic of fish oil encapsulated by the mixture of whey protein isolate and caseinate (ratio of 4:1). At the same outlet temperature, a higher inlet temperature decreased the microencapsulation efficiency of microcapsules. At inlet temperature of 170 °C and outlet temperature of 70 °C exhibited the highest microencapsulation efficiency (48.9%) (Chen *et al.*, 2013).

C Air outlet temperature

Air outlet temperature, obtained under given conditions, can be considered as the control index of the dryer. It is quite difficult to predict this outlet temperature in advance for a given product, since it depends on the drying characteristics of the material (Gharsallaoui *et al.*, 2007). Contrary to the air inlet temperature, the air outlet one cannot be directly controlled since it depends on the air

inlet temperature and the ideal air outlet temperature for the microencapsulation of food ingredients such as flavors has been reported to be 50–90 °C. The best spray-drying conditions are compromise between high air temperature, high solid concentration of the solution and easy pulverization and drying without expansion and cracks of final particles (Gharsallaoui *et al.*, 2007). Chen *et al.* (2013) reported that microencapsulation efficiency of encapsulated fish oils could be enhanced by drying at the optimum air outlet temperature (70 °C). This optimum condition also rendered smaller reconstituted particle size (0.35 μm) when compared to those prepared under air outlet temperature of 80 °C (1.0 μm). The lower microencapsulation efficiency and large reconstituted particle size might be due the extent of protein denaturation and aggregation, influenced by the higher outlet temperature.

1.2.6.5 Influence of encapsulation and antioxidant on lipid oxidation

In spray-dried emulsions, the amount of non-encapsulated oil is a key parameter determining the product quality (Drusch and Berg, 2008). Non-encapsulated oil or surface oil governs the flowability and wettability of spray-dried powders. Additionally, non-encapsulated core material is particularly prone to oxidation (Pourashouri *et al.*, 2013). For marine fish oils, autoxidation of polyunsaturated fatty acids leads to the development of volatile secondary oxidation products. For oxidized fish oil, 1-penten-3-one, (Z)-4-heptanal, 1-octen-3-one (Z)-1,5-octadien-3-one, (E,E)-2,4-heptadienal and (E,Z)-2,6 nonadienal have been identified as important volatiles (Venkateshwarlu *et al.*, 2004). Generally, the secondary lipid oxidation products may have a very low odor threshold and consequently negatively affect sensory property of the microcapsules (Venkateshwarlu *et al.*, 2004), limit consumer acceptance and shorten the shelf-life of these products.

To enhance the stability, the increase in wall concentration can be implemented, mainly related to the increased surface core prior to the formation of crust around the drying droplets (Gharsallaoui *et al.*, 2007). Furthermore, the incorporation of antioxidant could be another means to lower lipid oxidation. Olive lipid in the absence or presence of 300 ppm caffeic acid was encapsulated in 1.5% w/w sodium alginate. Encapsulated olive lipid with and without added caffeic acid

and unencapsulated olive lipid were stored at 20 or 37 °C for 30 days. It was found that caffeic acid addition increased the stability of the final lipid product. Oxidation was generally slower in the encapsulated lipid samples. Both encapsulation and addition of caffeic acid preserved unsaturated fatty acids (Sun-Waterhouse *et al.*). Baik (2004) reported that encapsulated fish lipid was 10 folds more stable against oxidation than the surface fish lipid. Tocopherol, which is a lipophilic antioxidant, showed a greater antioxidative effect in both surface and encapsulated fish lipid than ascorbyl palmitate, which is an amphiphilic antioxidant (Baik *et al.*, 2004). The lower PV and *p*-anisidine value and higher retention of EPA and DHA were found in encapsulated fish oil, compared to bulk fish oil and microcapsule containing fish oil, phytosterol and limonene (Chen *et al.*, 2013). The addition of phytosterol and limonene could reduce off-flavor and mask the fishy odor (Chen *et al.*, 2013). In the microencapsulated fish lipid, the addition of rosemary extracts rich in carnosic acid to ternary blends of tocopherols, ascorbyl palmitate and lecithin or Citrem significantly retarded autoxidation (Serfert *et al.*, 2009). Klinkesorn *et al.* (2005) studied the use of spray dryer to produce tuna oil in o/w emulsion encapsulated with lecithin and chitosan multilayer. The cationic interfacial membranes of lecithin and chitosan multilayer can retard lipid oxidation. The combination of ethylenediaminetetraacetic acid (EDTA) and mixed tocopherols was able to increase the oxidative stability of dried emulsions (Klinkesorn *et al.*, 2005). The influence of microencapsulation by spray drying and fluidization bed techniques on fish oil sensory quality was studied by Kolanowski *et al.* (2007). The fish oil coated with various materials (modified cellulose, skim milk powder and a mixture of fish gelatin and cornstarch) was spray-dried. Sample stored in the presence of air was highly susceptible to oxidation with increasing undesirable odor, especially in samples formed by spray-drying. Elimination of air from the packaging improved stability of all samples during storage in both odor profile and peroxide value. Therefore the microencapsulation produced by spray dryer provokes strong changes in fish oil sensory quality, especially in the presence of air. Its stability may be improved when stored under vacuum (Kolanowski *et al.*, 2007).

1.3 Objectives

1.3.1 To determine chemical compositions of tuna roes

1.3.2 To produce protein hydrolysates with antioxidative activity and to identify antioxidative peptides from the selected tuna roe

1.3.3 To study the application of roe protein hydrolysate as antioxidative emulsifier in fish sausage enriched with polyunsaturated fatty acids (PUFAs)

1.3.4 To investigate the application of tuna roe protein hydrolysate as emulsifier in oil in water emulsion prepared by different homogenization pressures

1.3.5 To study the use of tuna roe protein hydrolysate as wall material for encapsulating fish oil in the absence and presence of tannic acid or oxidized tannic acid

CHAPTER 2

Chemical compositions of the roes from skipjack, tongol and bonito

2.1 Abstract

Chemical compositions of roes from three tuna species including skipjack (*Katsuwonous pelamis*) (SJK), tongol (*Thunnus tonggol*) (TGL) and bonito (*Euthynnus affinis*) (BNO) were determined. Tuna roes contained 72.17-73.03% moisture, 18.16-20.15% protein, 3.29-5.68% lipid and 1.79-2.10% ash. Non-defatted and defatted roes from all species had the protein with a molecular weight of 97 kDa as the major component. Glutamic acid and glutamine were the most abundant amino acids (12.18-12.65 g/100g protein), whereas K, Na and P were the major elements in defatted tuna roes. Crude lipids from tuna roes contained phospholipids as the major component (51.22-54.90% of total lipids) with high content of docosahexaenoic acid (C22:6 n3) (20.53-26.19% of total lipids). Cholesterol contents of crude lipids from the roes of SJK, TGL and BNO were 172, 122 and 94 mg/100 g lipids, respectively. Therefore, tuna roe could be a promising source of valuable nutrients for human food and animal feeds.

2.2 Introduction

Roe is referred to oocyte gathered in skeins (Mahmoud *et al.*, 2008). During oocyte maturation or vitellogenesis, the precursor protein (vitellogenin) is transferred into oocytes and converted to lipovitellin and phosvitin by specific proteolytic cleavage. Lipovitellins and phosphoproteins, including phosvitins and phosvettes, are the major lipoprotein in fish egg yolk. Types and forms of yolk proteins may differ between different species of teleosts (Specker and Sullivan, 1994). Fish roe contains 11% albumins, 75% ovoglobulin and 13% collagen (Sikorski, 1994). Furthermore, roe has the high content of nutritive lipids, particularly phospholipids and long chain unsaturated fatty acids (LCUFAs) (Mahmoud *et al.*, 2008). *cis*-5,8,11,14,17-eicosapentaenoic acid (EPA; C20:5) and cis-4,7,10,13,16,19-docosahexaenoic acid (DHA; C20:6) play an important role in reduction of the risk of

coronary heart disease and prevention of Alzheimer's disease and anti-inflammatory disease (Sahena *et al.*, 2009). Roes from sturgeon and salmon, which are commonly consumed, contained high amount of proteins (26-29%) and lipids (5-10%) (Al-Holy and Rasco, 2006). Fish roes have the high content of polyunsaturated fatty acids (PUFAs) e.g. white sea bream (4.91-6.73% EPA and 23.09-27.50% DHA) (Cejas *et al.*, 2003), Pacific herring (13.72% EPA and 21.65% DHA) (Huynh *et al.*, 2007) and rainbow trout (11.5% EPA and 24.0% DHA) (Mahmoud *et al.*, 2008). Furthermore, roe protein hydrolysates had good functional properties such as solubility, emulsifying properties, fat adsorption capacity and foaming properties (Chalamaiah *et al.*, 2009; Sathivel *et al.*, 2009).

Thailand is the world's largest canned tuna producer and exporter. By the year 2008, tuna products with total amount of 483,894 tons and value of 61,036 million baht were exported (Department of Foreign Trade 2009). Tuna roe, a by-product generated from fish processing (approximately 1.5-3.0% of total weight), is generally used as animal feed or for pet food preparation in Thailand. Since fish roe has been reported as a source of protein and PUFAs (Bledsoe *et al.*, 2003), the recovery of those valuable components from tuna roe can increase its value and reduce waste disposal or treatment, thereby lowering environmental pollution. Nevertheless, a little information on chemical compositions of roes from skipjack, tongol and bonito, which are the economically important species for tuna canning in Thailand, exists. Thus the objective of this study was to determine chemical compositions of roe from those three tuna species.

2.3 Materials and methods

2.3.1 Chemicals

Chloroform and methanol were purchased from Lab-Scan (Bangkok, Thailand). Sodium dodecylsulfate (SDS) and β-mercaptoethanol (β-ME) were obtained from Sigma (St. Louis, MO, USA). Acrylamide, *N,N,N′,N′*-tetramethylethylenediamine (TEMED) and bis-acrylamide were procured from Fluka (Buchs, Switzerland).

2.3.2 Roe collection and preparation

Roes of skipjack (*Katsuwonous pelamis*) (SJK), tongol (*Thunnus tonggol*) (TGL) and bonito (*Euthynnus affinis*) (BNO) with the size of 57.7±2.3, 36.3±2.9 and 27.3±1.9 g/roe were obtained from Songkla Canning Public Co., Ltd., Songkhla, Thailand in November and December, 2009. The samples were placed in ice using a roe/ice ratio of 1:2 (w/w) and transported to the Department of Food Technology, Prince of Songkla University within 1 h. Roes (1 kg) were randomly taken, pooled and used as the composite samples. Three different composite samples from different batches were used in this study. The whole roes were cleaned using cool water (4°C) and powdered using a blender in the presence of liquid nitrogen. The powder obtained was placed in polyethylene bag, sealed and kept at -20°C until use.

2.3.3 Extraction of lipids

Lipids of tuna roes were extracted according to the method of Bligh and Dyer (1959). Samples (75 g) with a dry matter of approximately 20-21 g were homogenized with 150 mL of the mixture of chloroform: methanol (1:1, v/v) at 11,000 rpm using an IKA homogenizer (Model T25, Selangor, Malaysia) for 1 min. The homogenates were then added with 75 mL of chloroform and the mixtures were homogenized for 30 s at the same speed. The mixture was filtered through a Whatman No. 4 filter paper (Whatman International Ltd., Maidstone, UK.). The defatted roe was freeze-dried and kept at -20 °C until analysis.

For the filtrate, it was transferred into a separating funnel. The chloroform phase (bottom phase) was drained off into the Erlenmeyer flask. Sodium sulfate (anhydrous) (1-2 g) was added and the mixture was shaken thoroughly to remove the residual water. Lipid in chloroform was decanted into a rounded-bottom flask through a filter paper (Whatman No. 4). The chloroform was evaporated at 25 °C using a rotary evaporator (Rotavapor, model R-14, Buchi, Tokyo, Japan) and the residual solvent was removed by flushing nitrogen. The lipid was transferred to an amber vial and the sample was kept under nitrogen atmosphere at -20 °C until analysis.

2.3.4 Analyses

2.3.4.1 Proximate analysis

Samples, non-defatted and defatted roes, was subjected to proximate analysis including moisture, protein, fat and ash contents following the analytical No. of 950.46, 928.08, 960.39 and 920.153, respectively (AOAC, 2000).

2.3.4.2 SDS-polyacrylamide gel electrophoresis (SDS-PAGE)

Protein patterns of non-defatted and defatted roes were determined by SDS-PAGE using 4% stacking gel and 12% running gel according to the method of Laemmli (1970). To solubilize the samples, 27 mL of 5% SDS were added into 3 g of sample. 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. Proteins (15 μg) 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 separation, the proteins were stained with 0.02% (w/v) Coomassie Brilliant Blue R-250 in 50% (v/v) methanol and 7.5% (v/v) acetic acid for 3 h and destained with 50% (v/v) methanol and 7.5% (v/v) acetic acid for 15 min, followed by 5% (v/v) methanol and 7.5% (v/v) acetic acid for 3 h.

2.3.4.3 Amino acid analysis

For the determination of amino acid composition, the samples were prepared according to the method of Simpson *et al*. (1976). Defatted roes were hydrolyzed under reduced pressure in 4.0 M methanesulfonic acid containing 0.2% (v/v) 3-2(2-aminoethyl) indole at 115 °C for 24 h. The hydrolysates were neutralized with 3.5 M NaOH and diluted with 0.2 M citrate buffer (pH 2.2). An aliquot of 0.4 mL was applied to an amino acid analyser (MLC-703, Atto Co., Tokyo, Japan).

2.3.4.4 Determination of minerals

Iron (Fe), copper (Cu), manganese (Mn), magnesium (Mg), sodium (Na), phosphorus (P) and potassium (K) contents of defatted roes were determined by the inductively coupled plasma optical emission spectrophotometer, ICP-OES (Perkin Elmer, Model 4300 DV, Norwalk, CT, USA) according to the method of AOAC (1999). Samples (4 g) were mixed with 4 mL of concentrated nitric acid and shaked vigorously for 5 min. The mixtures were then heated using a hot plate until digestion was completed. The digested samples were transferred to a volumetric flask and the volume was made up to 10 mL with deionised water. The solution was subjected to ICP-OES analysis. Flow rates of argon to plasma, auxiliary and nebuliser were kept at 15, 0.2, and 0.8 L/min, respectively. Sample flow rate was set at 1.5 mL/min. The concentration of mineral was calculated and expressed as mg/kg sample.

2.3.4.5 Determination of sulfur content

Sulfur content was determined using CHNS-O analyser (Model Flash 1112 EA Series, CE Instruments, Milan, Italy). The sample (2-3 mg dry matter) housed in a tin capsule was dropped into a quartz tube at 900 °C with constant helium flow (carrier gas, flow rate 130 mL/min). Before the sample was dropped into the combustion tube, the stream was enriched with a measured amount of high purity oxygen (250mL/min) to achieve a strong oxidizing environment which guaranteed almost complete combustion/oxidation even of thermally resistant substances. The combustion gas mixture was driven through an oxidation catalyst (copper oxide, CuO) zone, then through a subsequent copper zone (electrolytic copper) in which sulfuric anhydride (SO_3) was eventually formed during combustion on catalyst reduction to sulfurous anhydride (SO_2) and the excessive oxygen was obtained. The resulting component of the combustion mixture was detected by a Thermal Conductivity detector (Thermoquest, CE Instruments, Milan, Italy).

2.3.4.6 Determination of lipid composition

Lipids of roes extracted as described by Bligh and Dyer (1959) were used for analysis. Lipid compositions were determined by thin layer

chromatography/flame ionisation detection analyser (TLC–FID) (IATROSCAN–TLC/FID Analyser, IATRON Laboratories, Inc., Tokyo, Japan). Scanned quartz rods (silica gel powder-coated Chromarod S III, IATRON Laboratories, Inc., Tokyo, Japan) were dipped in 3% boric acid solution for 5 min, dried and rescanned with the TLC–FID analyser. The sample solution (1 μL) was spotted on the rod and the separations were performed in the mixtures of benzene:chloroform:acetic acid (52:20:0.7) for approximately 35 min. Then the rods were dried in an oven (105 °C) for 10 min before being analysed with the FID detector. The analytical condition was H_2 flow rate of 150 mL/min, air flow rate of 2000 mL/min and scanning speed of 30 s/scan. Retention time of lipid composition standards including triolein, oleic acid, 1,3-diolein, 1,2-dioleyl-rac-glycerol, monopalmitin and phosphatidylcholine (Sigma Chemical Company, St. Louis, MO, USA) was used to identify chromatographic peaks. Peak area was quantitated and expressed as% of total lipid.

2.3.4.7 Determination of fatty acid compositions

Fatty acid compositions were determined as fatty acid methyl esters (FAMEs) using a gas chromatography, GC-14A (Shimadzu Co., Kyoto, Japan) equipped with fused silica capillary column Carbowax-30 m (30 m, 0.25 mm ID, Alltech Ltd., Deerfield, IL, USA) and flame ionisation detector (FID) (Alltech Ltd., Deerfield, IL, USA). Helium was used as the carrier gas at a flow rate of 30 cm/s. The initial temperature of the column was set at 170 °C and increased to 225 °C with a rate of 1 °C/min and then held at 220 °C for an additional 20 min. The detector temperature was set at 270 °C, while the temperature at the injection port was maintained at 250 °C. Retention time of FAME standards was used to identify chromatographic peaks. Peak area was quantitated and expressed as% of total saponifiable lipids (AOAC, 2000)

2.3.4.8 Determination of cholesterol content

The cholesterol content of crude lipids was determined according to the method of Lee *et al.* (1998) with a slight modification. Lipids were methylated with tetramethylammonium hydroxide and subjected to GC analysis. The analysis of

cholesterol was conducted using HP-innowax capillary column (10m, 0.10 mm I.D.) (Agilent Technologies, Inc., Palo Alto, CA, USA) connected to Agilent Technology model 6890N Gas Chromatography (Agilent Technologies, Inc., Palo Alto, CA, USA) fitted with a flame ionisation detector (Agilent Technologies, Inc., Palo Alto, CA, USA). High purity helium was used as the carrier gas set at 1.0 mL/min and the sample (1µL) was injected into the GC. The temperature at injector port was set at 275 °C. Split/Splitless injection mode (10:1 for each injection) was used. Oven temperature was set at 250 °C and hold for 15 min and the detector temperature was set at 300 °C. The content of cholesterol for each sample was calculated from peak area and expressed as mg/100g lipids.

2.3.5 Statistical analysis

All experiments were run in triplicate. All analyses were conducted in triplicate, except for amino acid and fatty acid composition analyses, which were performed in duplicate. Statistic analysis was performed using one-way analysis of variance (ANOVA). Mean comparison was carried out using Duncan's multiple range test (Steel and Torrie, 1980). Analysis was performed using the SPSS package (SPSS for windows, SPSS Inc., Chicago, IL, USA).

2.4 Results and discussion

2.4.1 Proximate compositions

Proximate compositions of roes from three different tuna species are shown in Table 5. All roes contained 72.17-73.03% moisture, 18.16-20.15% protein, 3.39-5.68% lipid and 1.79-2.10% ash. No differences in moisture and ash contents were observed among all roes ($p > 0.05$). SJK roe had the highest protein content with the lowest lipid content, compared with those from TGL and BNO ($p < 0.05$). The highest lipid content was found in TGL roe (5.68%), followed by BNO roe (4.26%) and SJK roe (3.39%), respectively. Bledsoe *et al.* (2003) reported that roes of marine fish species including Alaska pollock (*Theragra chalcogramma*) and cod (*Gadus morhua*) roes contain 67.4-80.0% moisture, 16.0-25.8% protein, 0.3-5.2% lipid and 1.7-2.3% ash. Roes of freshwater fish species including catla (*Catla catla*), carp

(*Cyprinus carpio*), rohu (*Labeo rohita*) and murrel (*Channa striatus*) consisted of 50.7-67% moisture, 16.6-28.2% protein, 3.2-9.5% fat and 1.1-1.4% ash (Balaswamy *et al.*, 2009). The catfish (*Ictalurus punctatus*) roe contained 55.7% moisture, 28.1% protein, 16.5% fat, and 1.8% ash (Sathivel *et al.*, 2009). The result indicated that moisture content of tuna roes was comparable to those of Alaska pollock and cod roes, but was higher than those of roes from freshwater species. Similar fat content was found between tuna roes (3.39-5.68%) and roes from other species e.g. Alaska pollock (5.2%), catla (3.9%), carp (3.2%) and rohu (5.3%).

Table 1 Proximate compositions of the roes from skipjack, tongol and bonito

Compositions (% wet basis)	SJK	TGL	BNO
Moisture	72.17±0.67[†a‡]	72.23±0.96[a]	73.03±0.71[a]
Protein	20.15±.0.49[a]	18.44±0.27[b]	18.16±0.91[b]
Lipid	3.39±0.56[c]	5.68±0.11[a]	4.26±0.05[b]
Ash	1.94±0.11[a]	2.10±0.29[a]	1.79±0.25[a]
Carbohydrate	2.35±0.13[b]	1.55±0.11[c]	2.76±0.21[a]

SJK: skipjack (*Katsuwonous pelamis*); TGL: tongol (*Thunnus tonggol*); BNO: bonito (*Euthynnus affinis*)

† Values are mean ± SD (n=3)

‡ Different lowercase superscripts in the same row indicate significant differences ($p < 0.05$)

When the roe undergoes maturation, the ratio of moisture/lipid in the roe generally increases (Bledsoe *et al.*, 2003). The variation in chemical composition of fish roes is mainly attributed to biological factors including species, maturity stages, diet, season, harvest area and processing condition (Mahmoud *et al.*, 2008). Furthermore, quantity and compositions of fish lipids vary with species and habitats (Sahena *et al.*, 2009). Therefore, tuna roes could be an alternative source of proteins and lipids.

2.4.2 Protein patterns

Protein patterns of non-defatted and defatted tuna roes are shown in Figure 4. Protein with a molecular weight (MW) of 97 kDa was dominant in roes of SJK and BNO. Proteins with MW of 97, 66 and 55 kDa were predominant in TGL roe. The protein with a MW of 97 kDa might be a vitellin-like protein, which was found in salmon (*O. keta*) and sturgeon (*Acipencer transmontanus*) roes (Al-Holy and Rasco, 2006). Proteins with MW of 50-65 kDa were also observed in all roes. The protein bands with MW of 55-66 kDa might be subunits of vitelline envelope protein (Darie *et al.*, 2004).

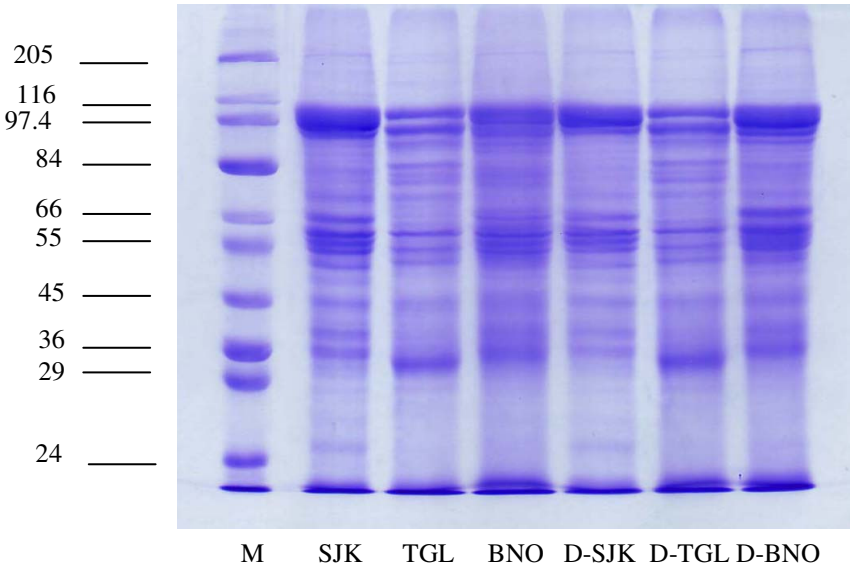


Figure 1 Protein patterns of non-defatted and defatted roes from three tuna species. M: molecular weight marker; SJK: skipjack; TGL: tongol; BNO: bonito; D-SJK: defatted-skipjack; D-TGL: defatted- tongol; D-BNO: defatted-bonito

Furthermore, proteins with MW of 32.5, 29 and 32.5 kDa were found in SJK, TGL and BNO roes, respectively. Those proteins might be ovomucoid (Al-Holy and Rasco, 2006) or phosvitin (Losso *et al.*, 1993). Generally, different roe samples showed different electrophoretic patterns, indicating the differences in protein compositions among all samples. Albumins and ovoglobulins constitute about 11% and 75% of total nitrogen of fish roe. Collagen content of fish roe is about 13% of the crude protein (Sikorski, 1994). Vitelline envelope (VE) of rainbow trout

(*Oncorhynchus mykiss*) roe is composed of 3 proteins involving VEalpha (58 kDa), VEbeta (54 kDa), and VEgamma (47 kDa) (Darie *et al.*, 2004). Losso *et al.* (1993) suggested that proteins with MW between 10 and 30 kDa in chicken and salmon roe could be phosvitin. Three prominent proteins of salmon caviar had MW of 96, 20 and 10 kDa, which colud be vitellin and possibly lysozyme or phosvitin. Protein with a MW of approximately 27 kDa in the soluble fraction of sturgeon caviar may possibly represent ovomucoid, a glycoprotein, which normally has a MW of 27–29 kDa (Al-Holy and Rasco, 2006). During vitellogenesis and oocyte maturation, barfin flounder (*Verasper moseri*) oocytes had the lipovitellins derived from vitellogenins A and B, which play distinct roles in regulation of egg buoyancy (Matsubara *et al.*, 2003). Lipovitellins are composed of distinct heavy chains (107 and 94 kDa) and light chains (30 and 28 kDa) (Matsubara *et al.*, 1999). Therefore, it was suggested that roes from three tuna species were most likely in the stage of final maturation. When comparing the protein pattern of roe under reducing and non-reducing conditions (data not shown), no marked difference was noticeable between both conditions. It was more likely that disulfide bonds were not mainly involved in stabilization of proteins in tuna roes.

2.4.3 Amino acid composition

After defatting, proteins in roe became concentrated and defatted roe could be used as proteinaceous sources for further application. Defatted-tuna roes (D-SJK, D-TGL and D-BNO) contained different amino acid compositions (Table 6). The major non-essential amino acids of all samples were glutamic acid /glutamine (12.18-12.65 g/100g protein) and aspartic acid/asparagine (8.27-8.85 g/100g protein). Leucine (8.28-8.64 g/100g protein) and lysine (8.24-8.30 g/100g protein) were the predominant essential amino acids in defatted tuna roes. Total essential amino acid (arginine, histidine, isoleucine, leucine, lysine, methionine, phenylalanine, threonine, valine) content of all samples was lower than total non-essential amino acid content. Negligible content of cysteine and hydroxylysine were found in all defatted-tuna roes. The higher hydroxyproline content was observed in D-BNO roe, compared with that of other defatted tuna roes. D-TGL roe contained the lower content of imino acids (proline and hydroxyproline) than did D-SJK and D-BNO roes. Glycine content was

higher in D-BNO roe than D-SJK and D-TGL roes, while no difference in glutamic acid/glutamine was observed among all three samples. However, the different amino acid composition of tongol roe was found by Zieaian *et al.* (2008).

Table 2 Amino acid compositions of the defatted roes from skipjack, tongol and bonito

Amino acid (g/100g protein)	D-SJK	D-TGL	D-BNO
Ala	6.80	6.94	6.66
Arg	6.87	6.50	6.96
Asp/Asn	8.53	8.27	8.85
Cys	0.04	0.04	0.05
Glu/Gln	12.65	12.18	12.55
Gly	4.27	6.22	5.57
His	2.84	3.14	2.91
Ileu	4.94	5.27	4.69
Leu	8.44	8.64	8.28
Lys	8.30	8.26	8.24
Hyl	0.04	0.04	0.20
Met	2.76	2.64	2.71
Phe	4.57	4.43	4.37
Hyp	0.14	0.13	0.75
Pro	6.17	5.29	5.85
Ser	6.16	5.96	5.69
Thre	4.85	4.93	4.92
Tyr	4.68	4.46	4.20
Trp	1.16	1.10	1.13
Val	5.80	5.57	5.43
EAA	49.37	49.38	48.51
NEAA	50.63	50.62	51.49
EAA/NEAA	0.98	0.98	0.94

D-SJK: defatted skipjack; D-TGL: defatted tongol; D-BNO: defatted bonito
EAA: essential amino acids; NEAA: non-essential amino acids.

Zieaian *et al.* (2008) reported that dominant essential amino acids of tongol roe are histidine (10.80 g/100g protein), leucine (10.30 g/100 g protein) and lysine (11.70 g/100 g protein), whereas predominant non-essential amino acids are glutamic/glutamine (5.89 g/100 g protein), proline (3.79 g/100 g protein) and serine (3.23 g/100 g protein). The different in amino acid composition of defatted tongol roe

and fresh tongol roe reported by Ziaeian *et al.* (2008), might be due to the different habitat, feeding and season. Glycine and alanine contribute to sweetness, whereas arginine is associated with bitterness. Glutamic and aspartic acid exhibit the umami taste (Osako *et al.*, 2007). It was more likely that proteins from defatted tuna roes might be used as the proteinaceous flavourants. Amino acid compositions of defatted tuna roe powders were similar to those of mullet, cod, pollock and chinook salmon (Bekhit *et al.*, 2009; Bledsoe *et al.*, 2003). Thus, protein rich defatted tuna roes could be an alternative source of amino acids with nutritive value.

2.4.4 Mineral content

Mineral (K, Na, P, Fe, Cu, Mn and Mg) contents of defatted-tuna roes are presented in Table 7. K was the most dominant mineral in all tuna roes, followed by P, Na and Mg, respectively. Fe, Cu and Mn were found as the minor minerals. For different defatted tuna roe, varying mineral content was observed, depending on species.

Table 3 Mineral content of the defatted roes from skipjack, tongol and bonito

Minerals (mg/kg, dry basis)	D-SJK	D-TGL	D-BNO
K	2397.56±36.44[†a‡]	2184.22±7.43[b]	2194.34±51.35[b]
Na	1074.93±22.14[a]	826.26±10.99[b]	768.25±12.14[c]
P	1502.98±17.28[ab]	1527.71±27.50[a]	1456.63±14.86[b]
Fe	70.22±0.22[c]	55.24±0.78[b]	122.17±0.88[a]
Cu	12.72±0.15[b]	12.48±0.07[b]	34.35±0.45[a]
Mn	0.34±0.01[b]	0.80±0.02[a]	0.78±0.01[a]
Mg	456.91±5.35[c]	691.46±6.08[a]	486.33±5.45[b]

D-SJK: defatted skipjack; D-TGL: defatted tongol; D-BNO: defatted bonito

† Values are mean ± SD (n=3)

‡ Different lowercase superscripts in the same row indicate significant differences ($p < 0.05$)

D-SJK roe had the higher K and Na contents, compared with D-TGL and D-BNO roes ($p < 0.05$). Nevertheless, D-BNO roe contained the higher contents of Fe, Cu and Mn than did D-SJK and D-TGL roes ($p < 0.05$). The highest Mg content was found in D-TGL roe ($p < 0.05$). P has been generally implied to the phospholipid content and the presence of phosphoprotein (Mahmoud *et al.*, 2008; Matsubara *et al.*, 2003). Fe and Cu are classified as the essential trace elements required for physiological and metal metabolic processes of marine organisms (Thanonkaew *et al.*, 2006). The excess intake of Fe and Cu can be detrimental to human health. The toxic limit of Cu is 30 mg/kg (FAO, 1983). Nevertheless, metal ions (Fe, Cu, Mn and Mg) in defatted roe might serve as the catalyst for lipid oxidation. Metal ions have been shown to exhibit the prooxidation activity (Thanonkaew *et al.*, 2006). Similar K, P and Na contents in defatted tuna roes were found in comparison with those of salmon roe (Bekhit *et al.*, 2009). However, Fe and P contents of all samples were lower than those of catla, carp and rohu roes (Balaswamy *et al.*, 2009). Different contents of elements were found among all defatted tuna roes. Different amount and types of elements were suggested to be governed by the maturation stages of roes (Bekhit *et al.*, 2009). Those tuna roes could serve as the essential source of minerals for improving human or animal nutrition.

2.4.5 Sulfur content

Roes of D-TGL and D-BNO contained sulfur (S) at a level of 8,800 mg/kg (dry basis), whereas D-SJK roe had S content of 10,800 mg/kg. Sulfur, measured by CHNS-O-analyser, represented in term of organic, inorganic and polymeric materials. Methionine (Table 6) partially contributed to S content of defatted tuna roes. Bekhit *et al* (2009) reported that salmon roe had S content of 1647-2443 mg/kg (wet basis). Catfish roe protein powder had S content of approximately 0.56 ppm (Sathivel *et al.*, 2009). The result implied that tuna roe had high content of sulfur containing compounds, which could undergo the decomposition during storage, causing the off-odor. Dimethyl sulfide and other sulfur compounds are derived from degradation of sulfur-containing amino acids and are associated with irradiated odor formation (Brown *et al.*, 1986). Brown *et al.* (1986) reported the odor of dimethyl

sulfide as sulfurous, or "bad eggs," and was associated with spoilage of egg components.

2.4.6 Lipid class

Lipid compositions of tuna roes determined by TLC-FID are shown in Table 8. Phospholipids constituted as the major component, accounting for 50-55% of total lipids. No difference in phospholipid contents was noticeable among different tuna roes. Triglyceride was found as the second dominant lipid in tuna roes, ranging from 38.71 to 46.28%. Similar triglyceride content was found between all tuna roes. Lipids from SJK roe had the higher amount of free fatty acid and diglyceride contents in comparison with those from TGL and BNO roes.

Table 4 Compositions and cholesterol content of lipids from skipjack, tongol and bonito roes

Samples	Free fatty acid*	Diglyceride*	Triglyceride*	Phospholipid*	Cholesterol content (mg/100g lipid)
SJK	3.72±0.27$^{\dagger a\ddagger}$	2.67±0.32^{a}	38.71±4.09^{a}	54.90±4.49^{a}	172±0.53^{a}
TGL	2.16±0.69^{b}	1.39±0.55^{b}	45.61±6.76^{a}	50.92±7.83^{a}	122±0.35^{b}
BNO	1.11±0.16^{c}	1.39±0.39^{b}	46.28±6.32^{a}	51.22±6.62^{a}	94±0.14^{c}

SJK: skipjack (*K. pelamis*); TGL: tongol (*T. tonggol*); BNO: bonito (*E. affinis*)

*Expressed as percentage of total lipid

† Values are mean ± SD (n=3)

‡ Different lowercase superscripts in the same column indicate significant differences ($p < 0.05$)

Free fatty acid (FFA) formation could be attributed to the lipases and phospholipases in tuna roes. The phospholipid content of SJK, TGL and BNO roes was higher than that of roes from white sea bream (34.15%) (Cejas *et al.*, 2003) and rainbow trout (47.45%) (Mahmoud *et al.*, 2008). The increase in the proportion of polar lipids

generally related with maturation (Sahena *et al.*, 2009). Differences in the lipid compositions were reported among individual species (Bledsoe *et al.*, 2003; Sahena *et al.*, 2009).

2.4.7 Fatty acid profile

Fatty acid compositions of lipids from tuna roes are shown in Table 9. Crude lipids had a high content of polyunsaturated fatty acid (PUFAs) (33.38-39.68% of total saponifiable lipid), followed by saturated fatty acid (SFA) (28.46-33.76% of total saponifiable lipid) and monounsaturated fatty acid (MUFA) (19.48-21.51% total saponifiable lipid). Docosahexaenoic acid (DHA, C22:6 n3), an important n-3 fatty acid, was the dominant PUFAs in all samples, whereas the main SFA and MUFA were palmitic acid (16:0) and oleic acid (C18:1 n9), respectively. Contents of PUFAs in roe samples were in descending order: n-3>n-9>n-6 fatty acids. The high content of PUFAs is attributed to the nutrition requirement for embryonic and early larval development stages of marine fish (Cejas *et al.*, 2003).

For n-3 fatty acids in crude lipids from tuna roes, DHA (20-53-26.19% of total saponifiable lipid) was much higher than EPA (3.80-4.62% of total saponifiable lipid). The slightly difference in n-3 fatty acids in tongol roe has been reported by Ziaeian *et al.* (2008) in which DHA and EPA of tongol roe were 24.79 and 5.29% respectively. Ovaries and egg of white sea bream contained higher DHA (23.09-27.50%) than EPA (4.91-6.73%) (Cejas *et al.*, 2003). Both EPA and DHA were probably located in membrane phospholipid of different tissues. DHA presents in a much higher concentration than EPA in neural and visual cell membranes, particularly in rod cell outer segment membranes and synaptosomal membranes (Cejas *et al.*, 2003). Tuna oil from head also showed the much higher content of DHA (18.8-25.5%) than that of EPA (0.1%) (Chantachum *et al.*, 2000), while EPA content was greater than DHA content in oil from black sea bass egg (Seaborn *et al.*, 2009). The consumption of fish or fish oil containing n-3 PUFA has been found to be clinically beneficial to human health such as the alleviation of rheumatoid arthritis symptoms, protection against heart disease, improved brain and eye function in

infants (Boran *et al.*, 2006). However, the intake of fish roe containing high purine content has been suggested to avoid for Rheumatoid arthritis patients (Mortis, 2003).

Lipids from all tuna roes contained the low level of n-6 fatty acid (5.29-6.14% of total saponifiable lipid). Even though fish oils normally contain a negligible content of n-6 fatty acids (< 5% of total fatty acids) (Huynh *et al.*, 2007), they play a vital role in many biological functions such as osmoregulation, cardiovascular function, reproductive system, etc (Cejas *et al.*, 2003). Total content of n-6 PUFAs of lipids extracted from the roes of SJK, TGL and BNO were 5.87, 6.14 and 5.29% of total saponifiable lipid, respectively. Linoleic acid (C18:2 n6) ranged from 0.97 to 1.08% of total saponifiable lipid. The elongation and saturation of α-linoleic acid lead to synthesis of DHA and EPA in fish (Huynh *et al.*, 2007). Another important n-6 fatty acid, arachinodic acid (C20:4 n6), possesses a crucial biological function as a main precursor of various eicosanoids (Huynh *et al.*, 2007). The amount of arachinodic acid of crude lipids from all tuna roes ranged from 2.16 to 2.36% of total saponifiable lipid.

For n-9 fatty acid, oleic acid constituted as the most prevalent fatty acid, accounting for 10.66-12.81% of total saponifiable lipid. Oleic acid has been reported to play a role in energy metabolism for spawning fish during gonad development (Huynh *et al.*, 2007). The content of oleic acid is comparable to herring roe (12.28%), but lower than that of liver (38.14%) and intestine (31.31%) of non spawning herring (Huynh *et al.*, 2007).

For n-7 fatty acid, palmitoleic acid (C16:1 n7) and *cis*-vaccenic acid (C18:1 n7) were found at a low content in all samples (2.01-4.47% of total saponifiable lipid). Lipids from BNO roe had the higher content of both n-7 fatty acids than did those from SJK and TGL roes. Palmitoleic acid and *cis*-vaccenic acid are the ubiquitous minor components in animal tissue. Palmitoleic acid was demonstrated as lipokine, an adipose tissues-derived hormone involved in the regulation of insulin and systemic metabolic homeostasis associated with Type 2 diabetes, obesity, atherosclerosis and inflammatory disorders (Goldberg *et al.*, 2009).

Table 5 Fatty acid profile of lipids from skipjack, tongol and bonito roes

Fatty acids (% of total saponifiable lipid)	SJK	TGL	BNO
C14:0	1.68	1.68	1.89
C15:0	0.83	0.8	0.61
C15:1	0.63	0.69	0.76
C16:0	24.27	18.20	20.08
C16:1 n7	3.43	3.23	4.47
C17:0	1.01	1.39	0.89
C17:1	1.69	1.77	1.77
C18:0	5.70	6.15	7.34
C18:1 n9	12.81	11.00	10.66
C18:1 n7	2.01	2.41	3.50
C18:2 n6	0.97	1.10	1.08
C18:3 n3	0.53	0.56	0.64
C18:3 n6	0.27	0.29	0.28
C18:4 n3	0.56	0.48	0.61
C20:0	0.27	0.24	0.34
C20:1 n9	0.25	0.38	0.35
C20:2 n6	0.14	0.16	0.12
C20:3 n6	0.12	0.12	0.14
C20:4 n6 AA	2.16	2.36	2.18
C20:4 n3	0.35	0.37	ND
C20:5 n3 EPA	3.80	4.62	4.62
C22:4 n6	0.34	0.21	0.31
C22:5 n3	0.77	1.32	1.69
C22:5 n6	1.87	1.90	1.18
C22:6 n3 DHA	23.46	26.19	20.53
Unidentified peak	10.08	12.48	13.96
SFA	33.76	28.46	31.15
MUFA	20.82	19.48	21.51
PUFA	35.34	39.68	33.38
SFA/PUFA ratio	0.96	0.72	0.93
n-3	29.47	33.54	28.09
n-6	5.87	6.14	5.29
n-9	13.06	11.38	11.01
n-3/n-6 ratio	5.02	5.46	5.31

SJK: skipjack (*K. pelamis*); TGL: tongol (*T. tonggol*) ; BNO: bonito (*E. affinis*)

Among SFA, palmitic acid content was higher in lipid from SJK roe (24.27% of total saponifiable lipid), compared with those from BNO (20.08% of total saponifiable lipid) and TGL (18.20% of total saponifiable lipid) roes. Oleic acid, a dominant MUFA, constituted 10.66-12.81% of total saponifiable lipid. Palmitic acid and oleic acid were also found abundant in chinook salmon roe (palmitic acid: 11.65%; oleic acid: 4.32%) (Bekhit *et al.*, 2009) and white sea bream egg (palmitic acid: 19.06%: oleic acid: 4.71%) (Cejas *et al.*, 2003). Palmitic acid was a predominant source of potential metabolic energy in fish during growth and particularly during roe formation stage in female fish (Huynh *et al.*, 2007). Palmitoleic acid can be converted to *cis*-vaccenic by Delta9 desaturase and elongase, respectively. Palmitoleic acid has been found in other roes such as herring (4.00%) (Huynh *et al.*, 2007) and mature chinook salmon (4.54%) (Bekhit *et al.*, 2009). Tuna roe lipids had comparable *cis*-vaccenic acid content with mature chinook salmon (3.25%) (Bekhit *et al.*, 2009).

2.4.8 Cholesterol content

Crude lipids extracted from SJK, TGL and BNO roes consisted of cholesterol at levels of 172, 122 and 94 mg/100 g lipid, respectively (Table 8). All crude lipids showed the lower cholesterol content, compared with those of chum salmon roe (4,200 mg/100 g lipid) and cod roe (5,900-6800 mg/100 g lipid) (Bledsoe *et al.*, 2003). It was noted that lipid from SJK roe had the highest cholesterol content, whereas lipid from BNO roe showed the lowest cholesterol content. A recommendation for cholesterol intake is not more than 300 mg/day (Wardlaw and Insel, 1996). An excess cholesterol intake has been reported for increase in the risk of coronary heart disease (CHD) (Higuchi *et al.*, 2008). In general, fish roes are rich in cholesterol content (Cejas *et al.*, 2003; Higuchi *et al.*, 2008). However, the levels of cholesterol of lipids from SJK, TGL and BNO roes were lower than those of single chicken egg yolk (approximately 210 mg) and white sea bream roe (11,450 mg/100 g lipid) (Cejas *et al.*, 2003). Furthermore, the consumption of roe rich in DHA and EPA has been suggested to decrease plasma total cholesterol, triacylglycerol, phospholipid and glucose levels in mice (Higuchi *et al.*, 2008). Therefore crude oils or lipids of all tuna roes contained a lower cholesterol with the lower risk for health.

2.5 Conclusions

Roes from three species of tuna including skipjack, tongol and bonito were the promising sources of protein, fatty acid as well as minerals. Lipids were rich in DHA with the low cholesterol content. After defatting, the residue contained high protein with high content of essential amino acids. Tuna roes can be therefore used as proteinaceous raw materials for preparation of food ingredient or other uses.

CHAPTER 3

Antioxidative and functional properties of protein hydrolysate from defatted skipjack (*Katsuwonous pelamis*) roe

3.1 Abstract

Antioxidative and functional properties of protein hydrolysate from defatted skipjack (*Katsuwonous pelamis*) roe, hydrolyzed by Alcalase 2.4L (SRPH) with different degrees of hydrolysis (DH) at various concentrations were examined. As DH increased, the reduction of DPPH, ABTS radicals scavenging activities and reducing power were noticeable ($p < 0.05$). The increases in metal chelating activity and superoxide scavenging activity were attained with increasing DH ($p < 0.05$). However, chelating activity gradually decreased at DH above 30%. All activities except superoxide anion radical scavenging activity increased as the concentration of hydrolysate increased ($p < 0.05$). Hydrolysis using Alcalase could increase protein solubility to above 80% over a wide pH range (2-10). The highest emulsion ability index (EAI) and foam stability (FS) of hydrolysates were observed at low DH (5%) ($p < 0.05$). Concentrations of hydrolysates determined interfacial properties differently, depending on DH. The molecular weight distribution of SRPH with 5% DH was determined using Sephadex G-75 column. Two major peaks with the molecular weight of 57.8 and 5.5 kDa were obtained. Fraction with MW of 5.5 had the strongest metal chelating activity and ABTS radical scavenging activity. The results reveal that protein hydrolysates from defatted skipjack roe could be used as food additive possessing both antioxidant activity and functional properties.

3.2 Introduction

Enzymatic hydrolysis has been implemented to prepare protein hydrolysate in order to improve its functional properties, particularly solubility and to enhance bioactivity. The hydrolysis provides the shorter chain peptides with excellent solubility over a wide pH range. However, a very high degree of hydrolysis can have negative effects on the functional properties, especially interfacial properties

(Klompong *et al.*, 2007). Various commercial proteases such as Alcalase (EC 3.4.21.62), Flavourzyme (EC 3.4.11.1), Protamex (EC 3.4.21.62/ 3.4.24.28), Neutrase (EC 3.4.24.28), porcine pepsin (EC 3.4.23.1) have been used to produce hydrolysate with varying properties. Alcalase has been reported for its efficacy in preparation of fish protein hydrolysate from many sources (Chalamaiah *et al.*, 2009; Klompong *et al.*, 2007; Thiansilakul *et al.*, 2007a) and it showed higher proteolytic activity, compared with acid and neutral enzymes (Balaswamy, 2011; Klompong *et al.*, 2007). Generally, the obtained peptides have been found to possess bioactivity e.g. radical scavenging activity, calcium binding capacity, chelating (Klompong *et al.*, 2007), antihypertensive (Bougatef *et al.*, 2008), antiproliferative (Picot *et al.*, 2006), immunomodulating (Kitts and Weiler, 2003) and prolyl endopeptidase inhibitory activities (Sørensen *et al.*, 2004).

Fish roe is well known as a nutritive source for human consumption, particularly polyunsaturated fatty acids (PUFAs) (Intarsarisawat *et al*., 2011) and functional proteins such as vitellogenin and vitellogenin derivatives (lipovitellin and phosvitin). Those proteins are mainly located in egg yolk and naturally present as granule form of lipovitellin-phosvitin complex with low solubility (Castellani *et al.*, 2006). Hydrolysate from herring (*Clupea harengus*) gonad using Alcalase with good functional properties was prepared by Sathivel *et al.* (2003). Chalamaiah *et al.* (2009) reported that protein hydrolysates from underutilized meriga (*Cirrhinus mrigala*) fish egg prepared by Alcalase and papain exhibited high protein solubility over wide pH range (2-12) with good fat adsorption capacity, foam capacity and emulsifying capacity.

Tuna processing industry has been economically important for Thailand as the essential income generator. Among all tuna, skipjack has served as the most common species with the largest amount for canned tuna production in Thailand. During the butchering process, a large amount of roe has been collected and generally used as the animal feed with the low market value. To maximize the use of those byproducts, the hydrolysis can be implemented to enhance the functional properties and bioactivities, especially antioxidative activity, etc. The hydrolysate from tuna roe might possess the different characteristics and properties as determined

by the unique proteins and lipoprotein, etc. Nevertheless, there is a little information regarding protein hydrolysates from the roe of tuna and their antioxidative activity and functional properties as affected by degree of hydrolysis (DH) and concentration used. Therefore, the aim of this investigation was to study antioxidative activities and functional properties of protein hydrolysate from roe, a by product of skipjack tuna, a most commonly species used for canned tuna production in Thailand.

3.3 Materials and methods

3.3.1 Chemicals

Alcalase 2.4 L (2.4 units/mL) was obtained from Novo Nordisk (Bagsvaerd, Denmark). Nitro-blue tetrazolium salt (NBT), phenazine methosulfate (PMS), 1,1-diphenyl-2-picrylhydrazyl (DPPH), nicotinamide adenine dinucleotide (NADH) and 3-(2-Pyridyl)-5,6-diphenyl-1,2,4-triasine-*p*,*p'*-disulfonic acid monosodium salt hydrate (ferrozine), 2,4,6-trinitrobenzenesulfonic acid (TNBS), 2,2-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt (ABTS) and 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (Trolox) were purchased from Sigma Chemical Co. (St. Louis, MO). Sephadex G-75 was obtained from GE Healthcare UK Limited (Buckinghamshire, UK). All reagents were of analytical grade.

3.3.2 Preparation of defatted roe

Roe of frozen skipjack tuna with the size of 55-60 g/roe was obtained from Songkla Canning Public Co., Ltd., Songkhla, Thailand in November and December, 2010. Frozen whole skipjack tuna stored at -20 ºC for not longer than 1-2 months after capture were thawed using the running water. After butchering, the roes were collected, placed in polyethylene bag and stored at -20 ºC for not longer than 3 months. To prepare the defatted roe, the Bligh and Dyer method (1959) was used as previously described by Intarasirisawat *et al*. (2011).

3.3.3 Preparation of roe protein hydrolysate

Roe protein hydrolysate was prepared from defatted skipjack tuna roe using Alcalase. The defatted sample (2.5 g dry matter) was suspended in 95 mL of distilled water to obtain the protein concentration of 20 mg/mL. The mixture was preincubated at 50 °C for 20 min prior to enzymatic hydrolysis. The hydrolysis reaction was initiated by the addition of Alcalase at levels of 1 and 5% (w/w solid matter). The reaction was conducted at pH 8 and 50°C for up to 5 h. The mixture (1 mL) was taken at designated times (0, 5, 10, 20, 30, 40, 60, 90, 120, 150, 180, 240 and 300 min), mixed with 1 mL of 1% SDS solution (85°C) and then placed into a water bath at 85 °C for 15 min to terminate the enzymatic reaction and solubilize the sample. The hydrolysate obtained was subjected to the determination of degree of hydrolysis (DH). Hydrolysis time providing high DH was selected for further study.

The effect of Alcalase concentrations on DH of SRPH was studied. Different amounts of Alcalase (1, 2, 5, 6 and 10%, w/w solid matter) were added into the defatted roe suspension (2.5%, w/v) adjusted to pH 8.0 using 2 M NaOH and pre-incubated at 50 °C for 20 min. The mixtures were mixed thoroughly. The hydrolysis was preceded for the selected time (1 h) and enzyme was inactivated as previously described. SRPHs were determined for DH. Log_{10} (enzyme concentration) vs. DH was plotted. From the regression equation, the enzyme concentrations required to hydrolyze defatted tuna roe powder to obtain the desired DHs (5, 10, 20, 30, 40 and 50%) were calculated. SRPH samples with different DHs were freeze-dried using a SCANVAC CoolSafe™ freeze-dryer (CoolSafe 55, ScanLaf A/S, Lynge, Denmark). Samples were placed in polyethylene bag and kept at – 40 °C until analyses.

3.3.4 Determination of DH

DH of SRPH was determined according to the method of Benjakul and Morrissey (1997). SRPH samples with appropriate dilution (125 µL) were added with 2.0 mL of 0.2 M phosphate buffer (pH 8.2) and 1.0 mL of 0.01% TNBS solution. The solution was mixed thoroughly and placed in a temperature controlled water bath (Memmert, Bavaria, 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 mixtures were cooled at room temperature for 15 min. The absorbance was read at 420 nm and α-amino group was expressed in terms of L-leucine. The DH was defined as follows:

$$DH = [(L_s - L_0)/(L_{max} - L_0)] \times 100$$

where L_s is the amount of α-amino groups of hydrolysate sample. L_0 is the amount of α-amino groups in the original defatted roe. L_{max} is the total α-amino groups in the defatted roe obtained after acid hydrolysis (6 N HCl at 100 °C for 24 h).

3.3.5 Determination of antioxidative activities

Prior to determination of antioxidative activities, samples were dissolved in distilled water (pH 7.0).

3.3.5.1 DPPH radical scavenging activity

DPPH radical scavenging activity of SRPH was determined as described by Wu *et al.* (2003) with a slight modification. To 1.5 mL of sample solution with varying protein concentrations (5, 10 and 20 mg/mL), 1.5 mL of 0.15 mM DPPH in 50% ethanol were added. The mixture was mixed vigorously and allowed to stand at room temperature in the dark for 30 min. The absorbance of the resulting solution was measured at 517 nm using a UV-1601 spectrophotometer (Shimadzu, Kyoto, Japan). The blank was prepared in the same manner, except that distilled water was used instead of the sample. A standard curve was prepared using Trolox in the range of 10-60 μM. The activity was expressed as μmol Trolox equivalents (TE)/mL.

3.3.5.2 ABTS radical scavenging activity

ABTS radical scavenging activity of SRPH was determined as described by Thiansilakul *et al*. (2007a). The stock solutions included 14.8 mM ABTS solution and 5.2 mM potassium persulfate solution. The working solution was prepared by mixing two stock solutions in equal quantities and allowing them to react for 12 h at room temperature in dark. The solution was then diluted by mixing 1 mL

of ABTS solution with 50 mL of methanol in order to obtain an absorbance of 1.1 ± 0.02 units at 734 nm using a spectrophotometer. Fresh ABTS solution was prepared daily. Sample (150 µL) with different protein concentrations (5, 10 and 20 mg/mL) was mixed with 2850 µL of ABTS solution and the mixture was left at room temperature for 2 h in dark. The absorbance was then measured at 734 nm using a spectrophotometer. A standard curve of Trolox ranging from 50 to 600 µM was prepared. The activity was expressed as µmol Trolox equivalents (TE)/mL.

3.3.5.3 Superoxide radical scavenging activity

Superoxide radical scavenging activity was measured by the reduction of NBT according to the method of Hazra *et al.* (2008) with a slight modification. The superoxide radicals, generated by non-enzymatic phenasine methosulfate-nicotinamide adenine dinucleotide (PMS/NADH), reduce nitro blue tetrasolium (NBT) to a purple formazan. One millilitre of reaction mixture contained phosphate buffer (20 mM, pH 7.4), NADH (73 µM), NBT (50 µM), PMS (15 µM) and various sample concentrations (0-20 mg/mL). After incubation for 2 min at room temperature (28-30 °C), the absorbance at 562 nm was measured against a blank to determine the quantity of formazan generated. Trolox with the range of 0-600 µM was used as standard. Superoxide radical scavenging activity was expressed as µmol Trolox equivalents (TE)/mL.

3.3.5.4 Reducing power

Reducing power was determined by the method of Oyaizu (1986). The sample solution (0.5 mL) with different protein concentrations (5, 10 and 20 mg/mL) was mixed with 2.5 mL of 0.2 M phosphate buffer (pH 6.6) and 2.5 mL of 1% potassium ferricyanide. The mixture was incubated at 50 °C for 20 min. An aliquot (2.5 mL) of 10% trichoroacetic acid was added to the mixture, followed by centrifugation at 3,000 g (Hettich mikro 20, Tuttlingen, Germany) for 10 min. The upper layer of solution (2.5 mL) was mixed with 2.5 mL of distilled water and 2.5 mL of 0.1% ferric chloride and the absorbance was read at 700 nm. Trolox with the

concentration ranging from 0 to 600 μM was used as standard. Reducing power value was expressed as μmol Trolox equivalents (TE)/mL.

3.3.5.5 Metal chelating activity

The chelating activity on Fe^{2+} was determined using the method of Decker and Welch (1990). One millilitre of sample solution (5, 10 and 20 mg protein/mL) was mixed with 3.7 mL of distilled water. The mixture was then reacted with 0.1 mL of 2 mM $FeCl_2$ and 0.2 mL of 5 mM ferrozine for 20 min at room temperature. The absorbance was read at 562 nm. The blank was prepared in the same manner except that distilled water was used instead of the sample. EDTA with the concentration range of 0-3 μM was used as standard. Chelating activity was then calculated and expressed as μmol EDTA equivalent/mL.

3.3.6 Determination of functional properties

3.3.6.1 Solubility

Solubility was tested at different pHs following the method of Castellani *et al.* (2003) with a slight modification. Sample was dispersed in distilled water and pH was adjusted to different pHs (2.0, 3.0, 4.0, 5.0, 6.0, 7.0, 8.0, 9.0, 10.0) using either 6N HCl or 6N NaOH. The volume of the mixture was adjusted to desired volume to obtain the final concentration of 10 mg protein/mL using distilled water with corresponding pH. After stirring for 90 min, the mixtures were centrifuged at 1,800 g for 30 min using a Sorvall Model RC-B Plus centrifuge (Newtown, CT, USA) at room temperature. The supernatant was collected and subjected to determination of protein content using the Lowry's method (Lowry *et al.*, 1951)

3.3.6.2 Foaming properties

Foaming ability and foam stability of samples were determined according to the method of Shahidi *et al.* (1995). The samples were dissolved in distilled water (pH 7.0) to obtain designated protein concentrations (5, 10 and 20

mg/mL). Twenty millilitres of solutions were homogenized in a 50 mL-cylinder at a speed of 16,000 rpm to incorporate the air for 1 min. The total volume was measured at 0 and 30 min after whipping. Foaming ability was expressed as foam expansion at 0 min, while foam stability was expressed as foam expansion after 30 min of whipping. Foam expansion was calculated according to the following equation (Sathe and Salunkhe, 1981):

$$\text{Foam expansion (\%)} = [(A-B)/B] \times 100$$

$$\text{Foam stability (\%)} = [(A_{30\ min} - B)/(A_{0\ min} - B)] \times 100$$

where A = volume after whipping (mL) and B = volume before whipping (mL).

3.3.6.3 Emulsifying properties

Emulsion activity index (EAI) and emulsion stability index (ESI) were determined according to the method of Pearce and Kinsella (1978) with a slight modification. Samples at different protein concentrations (5, 10 and 20 mg/mL) were prepared using distilled water. Sample solution (6 mL) and soybean oil (2 mL) were homogenized using a homogenizer (model T25 basic, IKA LABORTECHNIK, Selangor, Malaysia) at a speed of 20,000 rpm for 1 min. Emulsions were pipetted out at 0 and 10 min and 100-fold diluted with 0.1% SDS. The mixture was mixed thoroughly for 10 s using a vortex mixer. The absorbance measured immediately (A_0) and after 10 min (A_{10}) of emulsification using a spectrophotometer were used to calculate EAI and ESI as follows (Pearce and Kinsella, 1978):

$$\text{EAI } (m^2/g) = (2 \times 2.303A)DF/l\phi C$$

$$\text{ESI (min)} = A_0 \times \Delta t/(A_0 - A_{10})$$

where A, A_{500}; l, path length of cuvette (cm); DF, the dilution factor (100); ϕ, oil volume fraction; C, protein concentration in aqueous phase (g/m^3) and Δt,10 min. The control was prepared by mixing distilled water and oil in the absence of sample.

3.3.7 Molecular weight distribution

SRPH with 5% DH, which exhibited the highest emulsifying properties and some antioxidative properties, was chosen to determine the molecular weight distribution using gel filtration chromatography. SRPH with 5% DH was dissolved in distilled water (mobile phase) to yield protein concentration of 50 mg/mL. The mixture was filtered through 0.45 μm Nylon filter membrane (25 mm, id.) (Xiboshi®, Tianjin, China). The filtrate (2 mL) was chromatographed on a Sephadex G-75 column (1.5 × 67 cm), equilibrated with approximately two bed volumes of elution buffer. Sample was loaded onto the column and then eluted with same buffer at a flow rate of 0.5 mL/min. Fractions of 6 mL were collected and their absorbance was monitored at 280 nm. The obtained fractions were subjected to determination of metal chelating activity and scavenging activity toward ABTS and superoxide anion radicals as previously described.

Molecular weight of SRPH (5% DH) was estimated by plotting the available partition coefficient (K_{av}) against the logarithm of the molecular weight of the protein standards. The elution volume (V_e) was measured for each protein standard and SRPH having 5% DH. The void volume (V_o) was estimated by the elution volume of blue dextran (M_r = 2000000). The low molecular weight markers included aprotinin (M_r = 6500), lysosyme (M_r = 14,600), soy bean trypsin inhibitor (M_r = 29 000), ovalbumin (M_r = 43000) and bovine serum albumin (M_r = 66000).

3.3.8 Statistic analysis

All experiments were run in triplicate. All analyses were conducted in triplicate, except for emulsion properties, which were performed in five replicates. Statistic analysis was performed using one-way analysis of variance (ANOVA). Mean comparison was carried out using Duncan's multiple range test (Steel and Torrie, 1980) Analysis was performed using the SPSS package (SPSS for windows, SPSS Inc., Chicago, IL, USA).

3.4 Results and discussions

3.4.1 Effect of Alcalase concentration and hydrolysis time on protein hydrolysis

Alcalase was the only protease, which was able to hydrolyze defatted skipjack roe, whilst Flavourzyme and Protamex could not hydrolyze roe proteins (data not shown). Chalamaiah *et al*. (2009) found that meriga fish egg hydrolysate prepared using Alcalase had higher DH (62% DH) than that using papain (17.1%). Alcalase has been reported to preferably hydrolyze hydrophobic amino acid residues (Chalamaiah *et al.*, 2009; Klompong *et al.*, 2007). Figure 5A displays DHs of hydrolysate from defatted skipjack roe (SRPH) as affected by hydrolysis time. DHs of SRPH gradually increased with increasing hydrolysis time ($p < 0.05$). Rapid hydrolysis was observed within the first 1 h. Thereafter, a slower rate of hydrolysis was found. A reduction in the hydrolysis rate might be due to the limitation of substrates for hydrolysis. The short chain peptides formed might not be cleaved by Alcalase. Decrease in hydrolysis rate could also be attributed to a decrease in peptide bonds available for hydrolysis, enzyme inhibition and deactivation (Dong *et al.*, 2008). An initial rapid phase was observed when a large number of peptide linkages were cleaved per unit time, rendering a large proportion of soluble protein. The most compact core proteins were hydrolyzed more slowly. At the same time, available substrate decreased as reaction time increased. The reduction of hydrolysis rate might be hypothetically attributed to a decrease in the concentration of peptide bonds available for hydrolysis, the decrease in enzyme activity or a product inhibition (Safari *et al.*, 2012). For the same hydrolysis time, the higher amount of enzyme used resulted in higher DH ($p < 0.05$). In the present study, hydrolysis time (1 h), whose the initial velocity was obtained, was selected for further study.

Different amounts of Alcalase were added to hydrolyze defatted skipjack roe for 1 h. When $\log_{10}$ (enzyme concentration) and DH were plotted, a linear relationship was observed (Figure 5B). From this regression, the exact concentration of Alcalase required to hydrolyze defatted skipjack roe to obtain

designated DH could be calculated. Alcalase is an alkaline microbial protease produced from *Bacillus licheniformis* (Chalamaiah *et al.*, 2009).

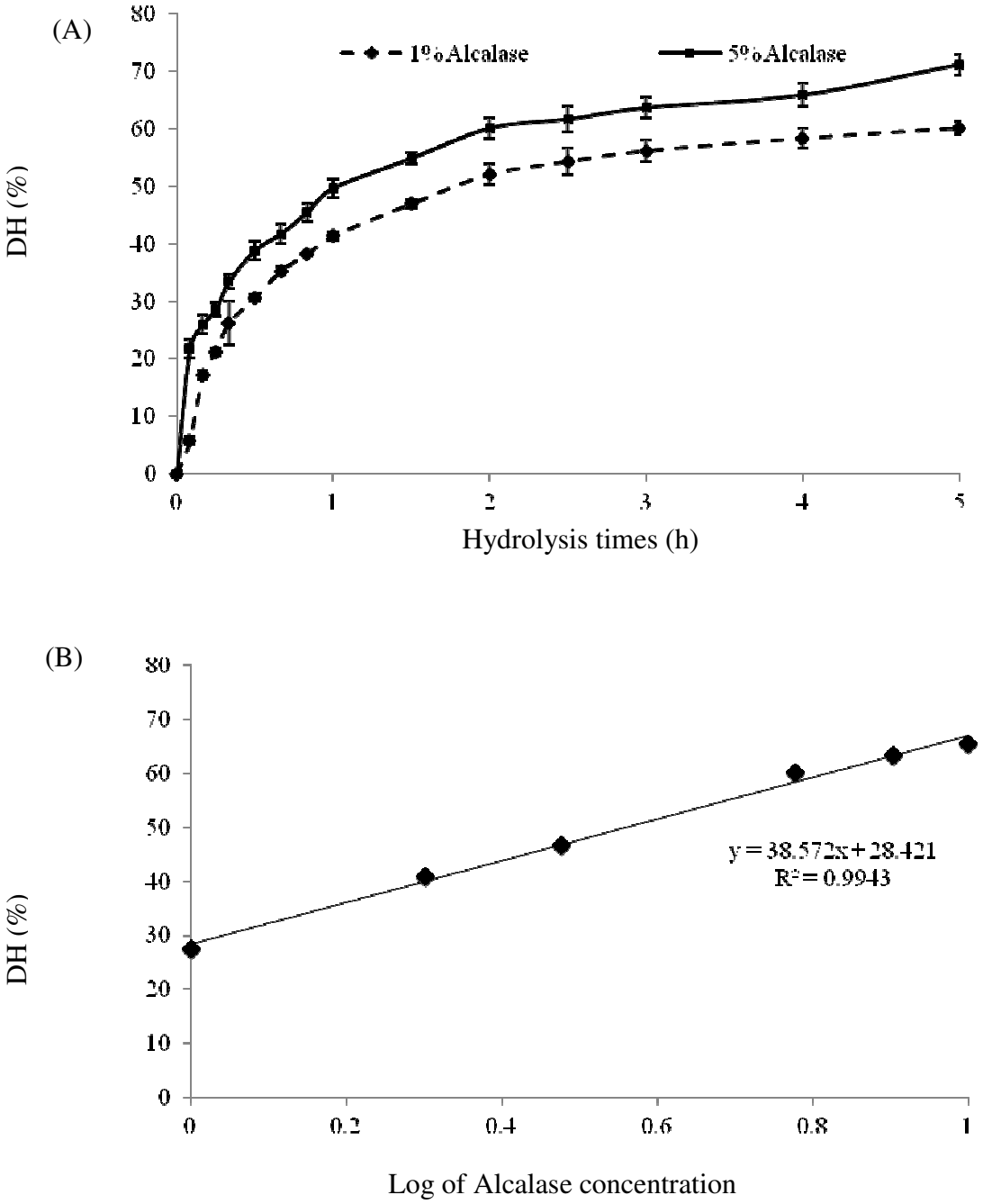


Figure 1 Degree of hydrolysis of defatted skipjack roe (2%, dry basis) hydrolyzed by 1 and 5% of Alcalase for various times (0-5 h) (A) and relationship between DH and log concentration of Alcalase for hydrolysis of defatted skipjack roe (B). The reaction was run for 1 h at pH 8.0, 50 °C

Alcalase has been successfully used to hydrolyze a variety of fish proteins such as underutilized meriga fish egg (Chalamaiah *et al.*, 2009), herring byproducts (Sathivel *et al.*, 2003), black scabbardfish (*Aphanopus carbo)* by-products and tuna fish by-product (Guerard *et al.*, 2002). At a level of 0.5% (w/w) Alcalase, meriga fish egg (6.5% substrate) reached 62% DH (Chalamaiah *et al.*, 2009), whilst 10.1% DH was found for herring gonad (50% substrate) (Sathivel *et al.*, 2003). Difference in DH among different proteinaceous materials used for hydrolysis was more likely determined by protein substrate and amino acid composition (Chalamaiah *et al.*, 2009; Sathivel *et al.*, 2003).

3.4.2 Antioxidative properties

Antioxidative properties of SRPH having different DHs (5-50%) at various concentrations (5-20 mg protein/mL) were evaluated using different assays (DPPH, ABTS, superoxide anion radical scavenging activities, reducing power and chelating capacity).

3.4.2.1 DPPH radical scavenging activity

DPPH radical scavenging assay has been widely used to evaluate antioxidative properties of compounds as free radical scavengers or hydrogen donors (Klompong *et al.*, 2007). SRPH (5-20 mg/mL) showed a scavenging effect on DPPH radical in a dose dependent manner ranging from 35 to 339 µmol TE/mL (Figure 6A). DPPH scavenging activity was also reported in protein hydrolysate prepared from yellow stripe trevally meat (Klompong *et al.*, 2007) and egg yolk (Sakanaka and Tachibana, 2006) prepared using Alcalase. Increasing amount of active peptides could react with free radicals to convert them to stable products more effectively (Batista *et al.*, 2010). At the same concentration used, the highest activity was found with SRPH having 5%DH (339 µmol TE/mL). As the DH increased, a decrease in activity was observed ($p < 0.05$). This might be governed by difference in chain length, amino acid composition, amino acid side chain (Klompong *et al.*, 2007) and hydrophobicity (Cheng *et al.*, 2010). Higher enzymatic cleavage resulted in the formation of shorter chain peptides possessing more hydrophilicity.

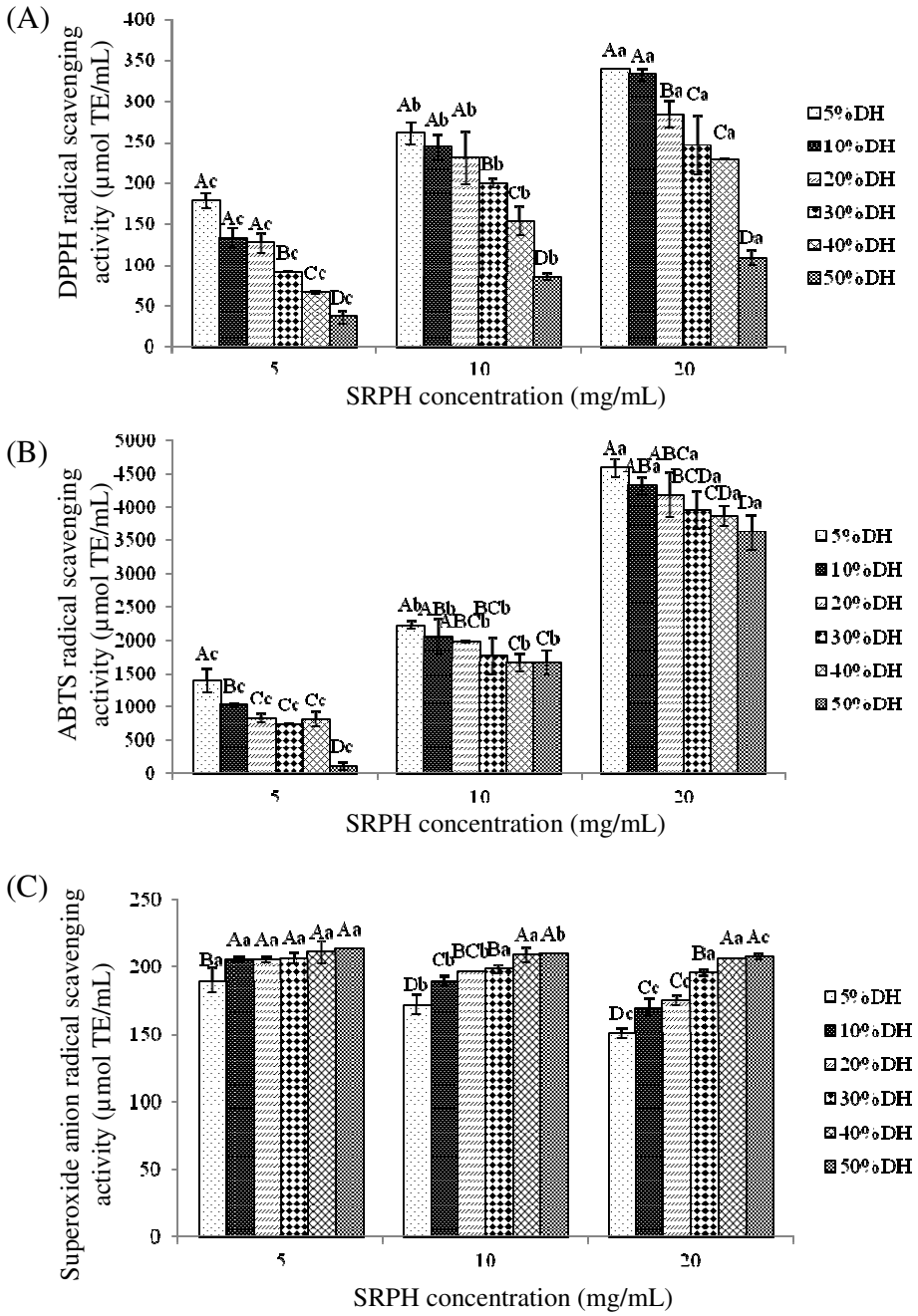


Figure 2 DPPH radical (A), ABTS radical (B), Superoxide anion radical (C) scavenging activities of hydrolysates from defatted skipjack roe with different DHs at varying concentrations. Bars represent standard deviations (n=3). Different uppercase letters within the same concentration indicate the significant differences ($p < 0.05$). Different lowercase letters within the same DH indicate the significant differences ($p < 0.05$).

Those peptides had less efficacy in scavenging $DPPH^{\bullet}$ radical, an oil - soluble free radical. Peptides from hydrolysate with higher DH could not interact properly with hydrophobic peroxyl radicals, due to the lack of hydrophobic domain of peptides (Cheng *et al.*, 2010). The antioxidant activity of protein hydrolysates also depends on the protease and hydrolysis conditions employed (Batista *et al.*, 2010). The results indicated that SRPH contained peptides acting as electron donors and free radical scavenger, thereby terminating the chain reaction.

3.4.2.2 ABTS radical scavenging activity

The assay of $ABTS^{+}$ radical scavenging activity can be applied to both lipophilic and hydrophilic compounds, and has been widely used as an antioxidant activity assay (You *et al.*, 2009). As shown in Figure 6B, SRPH samples at all DHs were able to sequester ABTS radical in a dose dependent manner ($p < 0.05$). At the same level, SRPH with 5% DH showed the highest activity ($p < 0.05$). Peptides in hydrolysate with higher DH might loss scavenging activity toward $ABTS^{+}$. Nalinanon *et al.* (2011) also reported that hydrolysates from ornate threadfin bream muscle having above 20% DH showed the decreased ability in scavenging ABTS radical. The amino acid sequence in peptides might affect antioxidant activity. Also, free amino acids produced determined the scavenging activity. Based on $ABTS^{\cdot+}$ scavenging assay, Cys was the most active amino acid, followed by Trp, Tyr and His (Gómez-Ruiz *et al.*, 2008). Peptides containing these amino acids might be responsible for higher ABTS radical scavenging activity. In general, similar results were obtained in comparison with those of DPPH radical scavenging activity (Figure 6A). Thus, the peptides in SRPH, particularly with 5% DH, were able to scavenge the radicals, thereby retarding the propagation stage. As a result, the lipid oxidation could be impeded in the presence of those hydrolysates.

3.4.2.3 Superoxide radical scavenging activity

The superoxide anion radical ($O_2^{\cdot-}$) scavenging capacity assay has been used to monitor the ability of hydrophilic antioxidants to directly react with this physiologically relevant radical (Moure *et al.*, 2006). The higher superoxide radical

scavenging activity was observed with increasing DH (Figure 6C). However, it was noted that there was difference in superoxide anion radical scavenging activity among SRPH having DHs from 10 to 50 at a low protein concentration (5 mg/mL). When the concentration of SRPH increased, superoxide anion radical generally decreased. This was possibly due to the self-aggregation of peptides induced by $O_2^{\cdot -}$, thereby lowering functional group to quench the tested radicals. Furthermore, this result indicated that the smaller peptides apparently showed higher superoxide anion radical scavenging activity than those having long chain length (Klompong *et al.*, 2007). Huang *et al.* (2010) reported that the purified peptide from egg shell protein hydrolysate with MW of 618.86 Da showed superoxide anion scavenging activity with IC_{50} at 0.10 mg/mL. Superoxide anion radical is an important reactive oxygen species (ROS) involving in the formation of highly reactive species such as hydroxyl radical and H_2O_2 which associates with cellular damage and implicated in aging process, numerous age-related degenerative diseases such as cancer, heart disease, macular degeneration, Parkinson's disease and chronic diseases (Li *et al.*, 2008). The results suggested that some peptides, especially those with the shorter chain length, exhibited the ability in scavenging or trapping the superoxide anion. As a consequence, the rate of lipid oxidation could be lowered in the presence of SRPH with the appropriate DH. The result suggested that peptides in SRPH exhibited the different scavenging mechanisms toward ABTS, DPPH and superoxide anion radicals.

3.4.2.4 Metal chelating activity

Metal chelating activity of SRPH as affected by DH and concentration is depicted in Figure 7A. At concentrations of 5 and 10 mg/mL, no differences in metal chelating activity were found when DH increased ($p > 0.05$). Nevertheless, chelating activity increased with increasing DH increased up to 40% ($p < 0.05$), when a concentration of 20 mg/mL was used. Furthermore, the marked decrease in metal chelating activity was found when DH was 50% ($p < 0.05$). Peptides with smaller size were not able to form the complex with metals. Xu *et al.* (2007) reported that phosvitin oligophosphopeptides, produced by partial dephosphorylation, followed by trypsin hydrolysis, had lower chelating activity than did phosvitin. Therefore, peptide chain length was more likely essential for chelating activity of SRPH. Steric

hindrance found in SRPH with longer chain might lower the ability to migrate and chelate the target metal ion. It was noted that chelating activity of all SRPHs was in a dose dependent manner. Phosvitin is a highly phosphorylated protein, which possesses strong metal chelating activity (Xu *et al.*, 2007). Metal chelating activity of phosvitin and derivatives is mainly related with phosphorus content and amino acid composition (Xu *et al.*, 2007). Additionally, their unique conformation, a blocks of phosphoserines in singular way, carrying up to 15 consecutive residues, is also reported as an ideal binding site for transition metals such as iron, copper, cobalt and essential minerals such as calcium and zinc (Castellani *et al.*, 2004). Transition metals in food could accelerate rate of autoxidation and breakdown of hydroperoxide to volatile compounds. Transition metal ions react very quickly with peroxides by acting as one-electron donors to form alkoxyl radical (Klompong *et al.*, 2007). Therefore, chelation of transition metal ions by antioxidant or antioxidative peptide resulted in the less availability of prooxidative metal ions. As a consequence the lipid oxidation could be retarded.

3.4.2.5 Reducing power

The reducing power assay is often used to evaluate the ability of an antioxidant to donate an electron or hydrogen (Klompong *et al.*, 2007). Antioxidative peptides in protein hydrolysate were able to reduce the Fe^{3+}/ferric cyanide complex to the ferrous form. Fe^{2+} complex can be monitored by measuring the formation of Perl's Prussian blue at 700 nm. The ability of SRPH with various DHs to reduce Fe^{3+} to Fe^{2+} was determined and expressed as reducing power in comparison with trolox (Figure 7B). All SRPH samples containing peptides showed reducing power in the range of 1307 - 5999 µmol TE/mL. The highest reducing power was obtained in SRPH having 5% DH ($p < 0.05$). Reducing power of all samples increased with increasing concentrations ($p < 0.05$). A little difference in reducing power among SRPHs with different DHs could be noticeable at protein concentration higher than 10 mg/mL. The results suggested that SRPH with excessive hydrolysis generally had lower reducing power. Reducing power of SRPH was also in accordance with DPPH - (Figure 6A) and ABTS-scavenging activities (Figure 6B). Therefore, the limited hydrolysis rendered SRPH with better antioxidative properties.

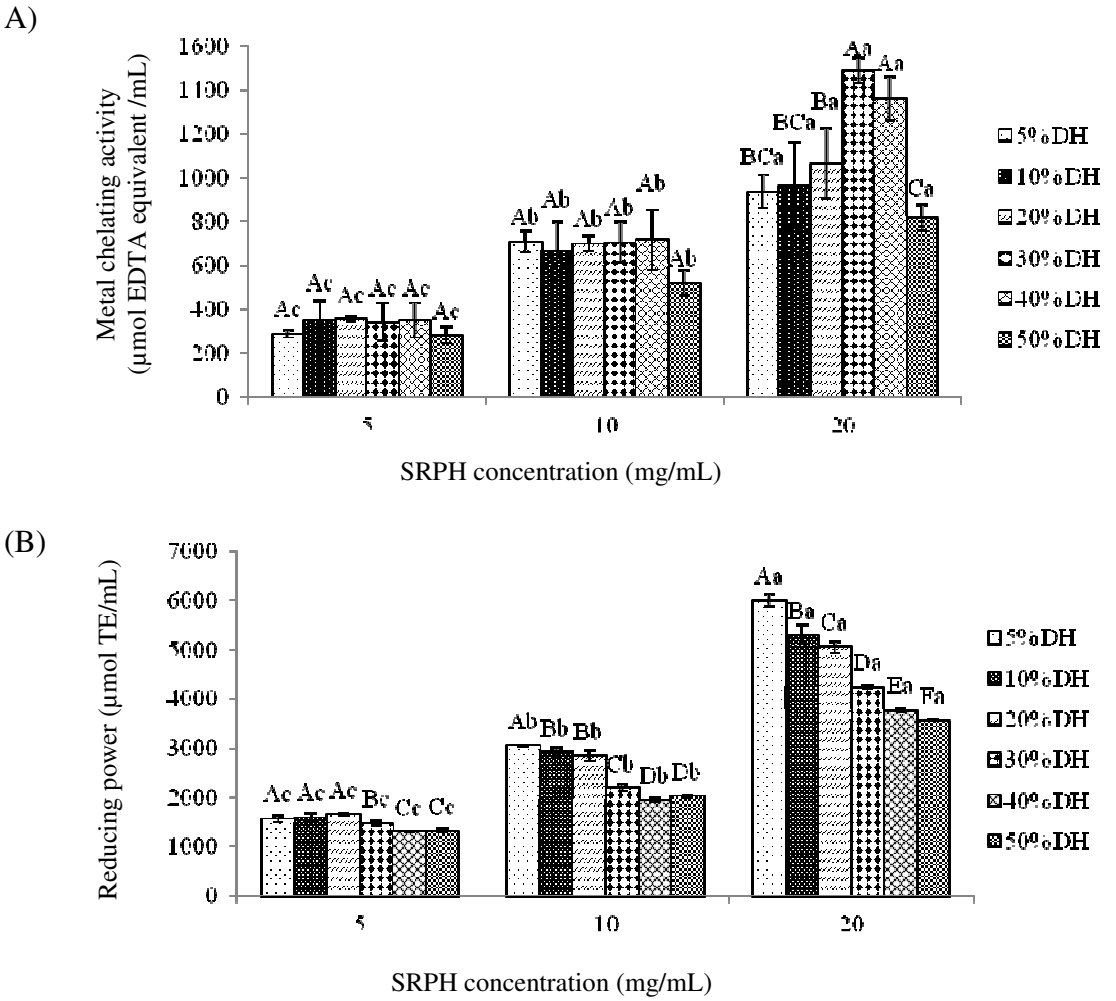


Figure 3 Metal chelating activities (A) and reducing powers (B) of hydrolysates from defatted skipjack roe with different DHs at varying concentrations. Bars represent standard deviations (n=3). Different uppercase letters within the same concentration indicate the significant differences ($p < 0.05$). Different lowercase letters within the same DH indicate the significant differences ($p < 0.05$).

Hydrolysate from yellow stripe trevally prepared using Alcalase had the decrease in reducing power when DH increased (Klompong *et al.*, 2007). On the other hand, hydrolysates prepared from several sources using varying enzymes such as black scabbard fish by-product (Batista *et al.*, 2010), loach (*Misgurnus anguillicaudatus*) meat (You *et al.*, 2009) and round scad (*Decapterus maruadsi*) protein hydrolysate (Thiansilakul *et al.*, 2007a) had higher reducing activity with increasing DH. The

difference in reducing power might be caused by different amino acid sequence and length of the obtained peptides (Thiansilakul *et al.*, 2007a). DH greatly influenced the peptide chain length as well as the exposure of terminal amino groups of products (Thiansilakul *et al.*, 2007a).

Therefore, SRPH possessed different modes of actions and DH was a major factor determining antioxidant activity. Additionally, the concentration was another factor influencing antioxidative activity.

3.4.3 Functional properties

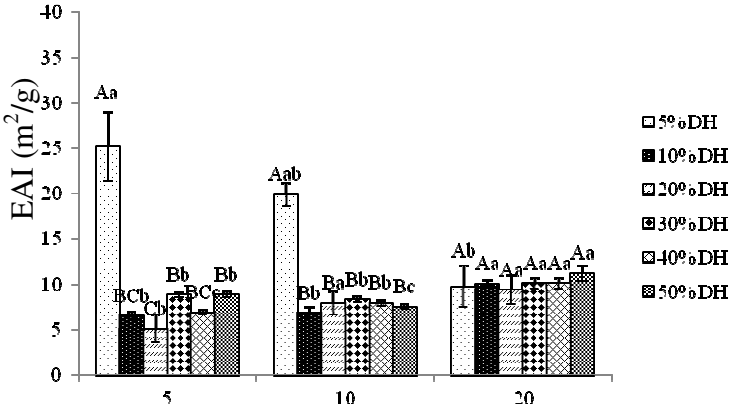
3.4.3.1 Protein solubility

Solubility is one of the most important functional properties of protein and can be increased by hydrolysis process (Klompong *et al.*, 2007). SRPH at various DHs (5-50%) was soluble over wide pH range with solubility of 82-99% (data not shown). Solubility of SRPH was higher than that of meriga egg hydrolysate (72 - 90%) (Chalamaiah *et al.*, 2009). The lowest solubility of all samples was noticeable at pH 3, whilst hydrolysate from meriga fish egg prepared with the aid of Alcalase showed the lowest solubility at pH 4. The lowest solubility at pH 3 was plausibly owing to the lowest repulsion of proteins at their pI. Phosvitin, a protein in fish roe, has pI of 4.0 (Castellani *et al.*, 2006). Enzymatic hydrolysis potentially affects the molecular size and hydrophobicity, as well as polar and ionisable groups of protein hydrolysates (Klompong *et al.*, 2007). The balance of hydrophilic and hydrophobic forces of peptides is another crucial influence on solubility (Gbogouri *et al.*, 2004; Klompong *et al.*, 2007). Generally, SRPH with higher DH tended to show higher solubility. The smaller peptides are expected to have proportionally more polar residues with the ability to form hydrogen bonds with water and augment solubility (Gbogouri *et al.*, 2004). As a consequence, hydrolysates with smaller peptides were more soluble. SRPHs with high solubility over wide pH range can be therefore applied widely in formulated food systems.

3.4.3.2 Emulsifying properties

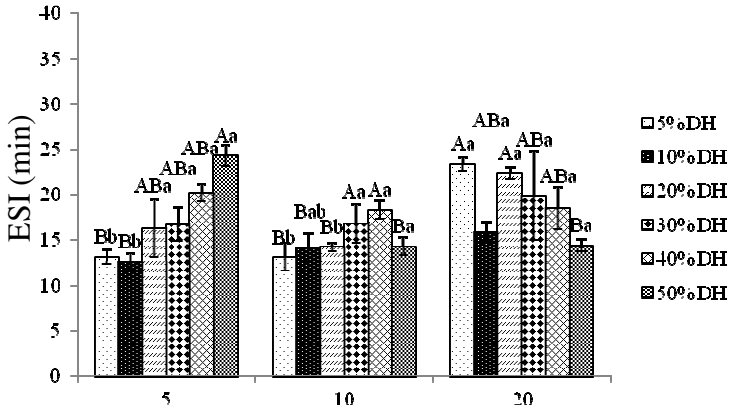
EAI and ESI of SRPH with different DHs at varying protein concentrations are depicted in Figure 8. EAI and ESI of SRPH were in the range of 5.1-25.16 m^2/g and 14.2-24.3 min, respectively. SRPHs with 5% DH showed the highest EAI ($p < 0.05$). No difference in EAI was found when DH was in the range of 10 - 50% ($p > 0.05$). EAI increased when protein concentration increased, regardless of DH ($p < 0.05$). Extensive hydrolysis commonly yielded the small peptides. Small peptides were more likely hydrophilic in nature. As a result, those peptides could not adsorb at the interface. They were more likely localized in the aqueous phase. Thus, the weaker EAI was generally observed as DH increased. As SRPH concentration increase, EAI of SRPHs having DH from 10 to 50% increased, whilst the decrease in EAI was observed for SRPH having 5% DH ($p < 0.05$). Peptides in SRPH with 5% DH more likely had the amphiphilic characteristic, which favored the migration of peptides to oil - water interface. The difference in EAI of SRPHs with different DHs might be associated with the varying chain length of their peptides. The excessive amount of protein hydrolysate might favor the self – aggregation of long chain peptides. As a result, the lower amount of peptides could localize at oil-water interface. Apart from peptide size, amphiphilicity of peptides is important for interfacial properties. When comparing with a random structural phosphorylated protein such as sodium caseinate (EAI = 10.3 - 39.8m^2/g; ESI = 20-99 min), it was found that all SRPH samples had lower emulsifying capacity and stability. Poorer emulsifying properties of SRPH sample might be owing to the loss of tertiary structure of protein (Sathivel *et al.*, 2003) and steric effect (Klompong *et al.*, 2007). Globular protein tends to form a highly elastic adsorbed layer, viscous solution and steric effect, contributing to higher emulsion stability (Sathivel *et al.*, 2004). Egg proteins complicating with emulsifying properties are phosvitin and lipoprotein, which could stabilize o/w emulsion mainly from electrostatic repulsion force (Castellani *et al.*, 2006).

(A)



SRPH concentration (mg/mL)

(B)



SRPH concentration (mg/mL)

Figure 4 Emulsion ability index (A) and emulsion stability index (B) of hydrolysate from defatted skipjack roe with different DHs at varying concentrations. Bars represent standard deviations (n=3). Different uppercase letters within the same concentration indicate the significant differences ($p < 0.05$). Different lowercase letters within the same DH indicate the significant differences ($p < 0.05$).

SRPH with 50% DH showed the highest ESI when determined at 5 mg/mL ($p < 0.05$). The similar ESI was observed between SRPH with different DHs

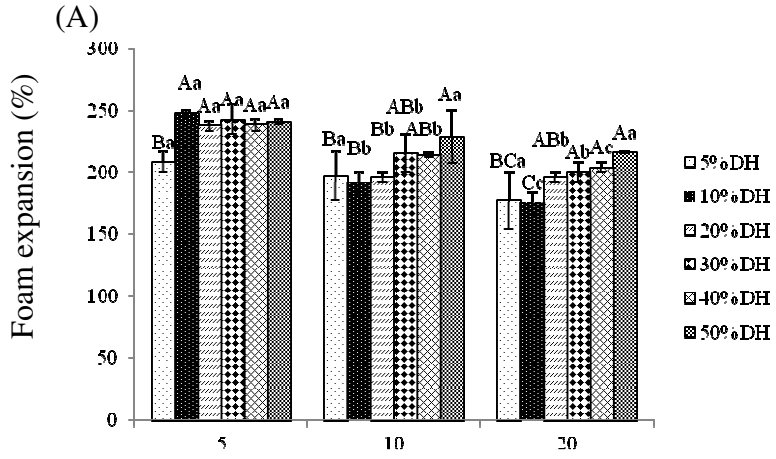
when tested at higher concentrations. However, ESI of SRPH possessing 50% DH was lower than that having 5% DH at a concentration of 20 mg/mL ($p < 0.05$). The hydrolysate might form the strong and thick film surrounding oil droplet. Nevertheless, the high charge density of phosvitin might result in a negligible adsorption capacity at o/w interface (Castellani *et al.*, 2006). It was noted that ESI varied with DH and concentration, suggesting that molecular property, chain length and amount of SRPH at the interface determined ESI of different SRPH.

3.4.3.3 Foaming properties

Foam expansion ability (FA) and foam stability (FS) after standing at room temperature for 30 min of SRPH with various DHs (5 - 50%) are shown in Figure 9. As DH increased, SRPH had higher FA ($p < 0.05$). The lowest FA was found with SRPH having 5% DH when tested at 5 mg/mL ($p < 0.05$). When DH increased, FA increased, depending on the concentrations used. Hydrolysate with high DH more likely had the smaller peptides, which could migrate to the air-water interface faster. With higher amount of peptides in SRPH, the lower FA was attained. The higher concentration of SRPH might result in the self aggregation and lowered the migration of peptides to the air - water interface. This was evidenced by the lower EAI with increasing concentration.

For foam stability, SRPH with 5% DH had the highest FS at all concentrations tested ($p < 0.05$). For all sample, FS remained unchanged when the concentration used increased, except for SRPH with 50% DH where FS increased with increasing concentration. It was found that SRPH with lower DH containing longer chain length yielded the foam with higher stability. This was plausibly due to the fact that peptides with the longer chain could form the thicker and stronger film surrounding air bubbles. Foam formation is governed by three factors, including transportation, penetration and reorganisation of molecules at the air–water interface (Klompong *et al.*, 2007). Protein requires to migrate rapidly to the air–water interface, unfolding and rearranging at the interface. Aewsiri *et al.* (2011) suggested that the foaming capacity of gelatin can be improved by incorporating fatty acid ester,

yielding more hydrophobic residues, which increased capacity of decreasing surface tension of foam.



(B)

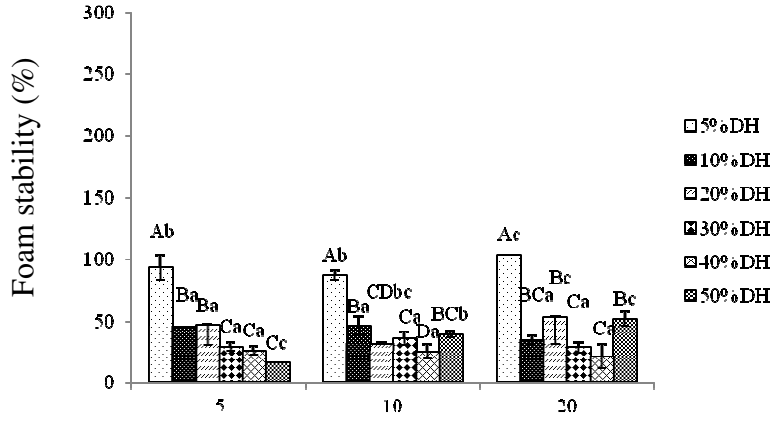


Figure 5 Foam ability (A) and foam stability (B) of hydrolysate from defatted skipjack roe with different DHs at varying concentrations. Bars represent standard deviations (n=3). Different uppercase letters within the same concentration indicate the significant differences ($p < 0.05$). Different lowercase letters within the same DH indicate the significant differences ($p < 0.05$).

3.4.4 Molecular weight distribution

Since SRPH having 5% DH displayed the highest antioxidative and functional properties when compared with others, it was determined for molecular weight distribution. After separation on Sephadex G-75, two main peaks of A_{280} with molecular weight (MW) of 57.8 (S1) and 5.5 kDa (S2) were obtained (Figure 10A). A_{280} indicates the presence of peptide, proteins, amino acids and more likely revealed the presence of tyrosine, tryptophan and other aromatic compounds in the peptides (Thiansilakul *et al.*, 2007a). Peak of S2 was larger than that of S1, suggesting the high proportion of low MW peptides in the SRPH (5% DH).

When the obtained fractions were measured for ABTS radical scavenging activity, superoxide anion radical and metal chelating activity, those fractions exhibited varying antioxidative activities. For metal chelating activity (Figure 10D) and ABTS radical scavenging activity (Figure 10B), peptides in S2 (F31-47) notably showed the stronger activity than S1 ($p < 0.05$). Peptides in selected fractions (F13-F43) could retard the generation of superoxide anion radical (Figure 10C). Low MW peptides possessing metal chelating activity and ABTS radical scavenging activity were generated by enzymatic hydrolysis. The enhanced exposure of the functional groups of proteins/peptides such as phosphoseryl group could favor their antioxidative activities. Balaswamy (2011) reported that roe protein hydrolysate from catla catla prepared using Alcalase yielded a high proportion of shorter peptides below 14 kDa. The peptides in the protein hydrolysates from meriga egg had an average MW of >10 kDa (Chalamaiah *et al.*, 2009). Hydrolysate from makerel mince with MW of 1400 Da had stronger antioxidative properties than that of small peptides (MW of 900 and 200 Da). Klompong *et al.* (2009b) reported that hydrolysates from yellow stripe trevally muscle with MW of 2.4 kDa exhibited higher antioxidative properties than those with MW of 35, 0.47 and 57 kDa, respectively.

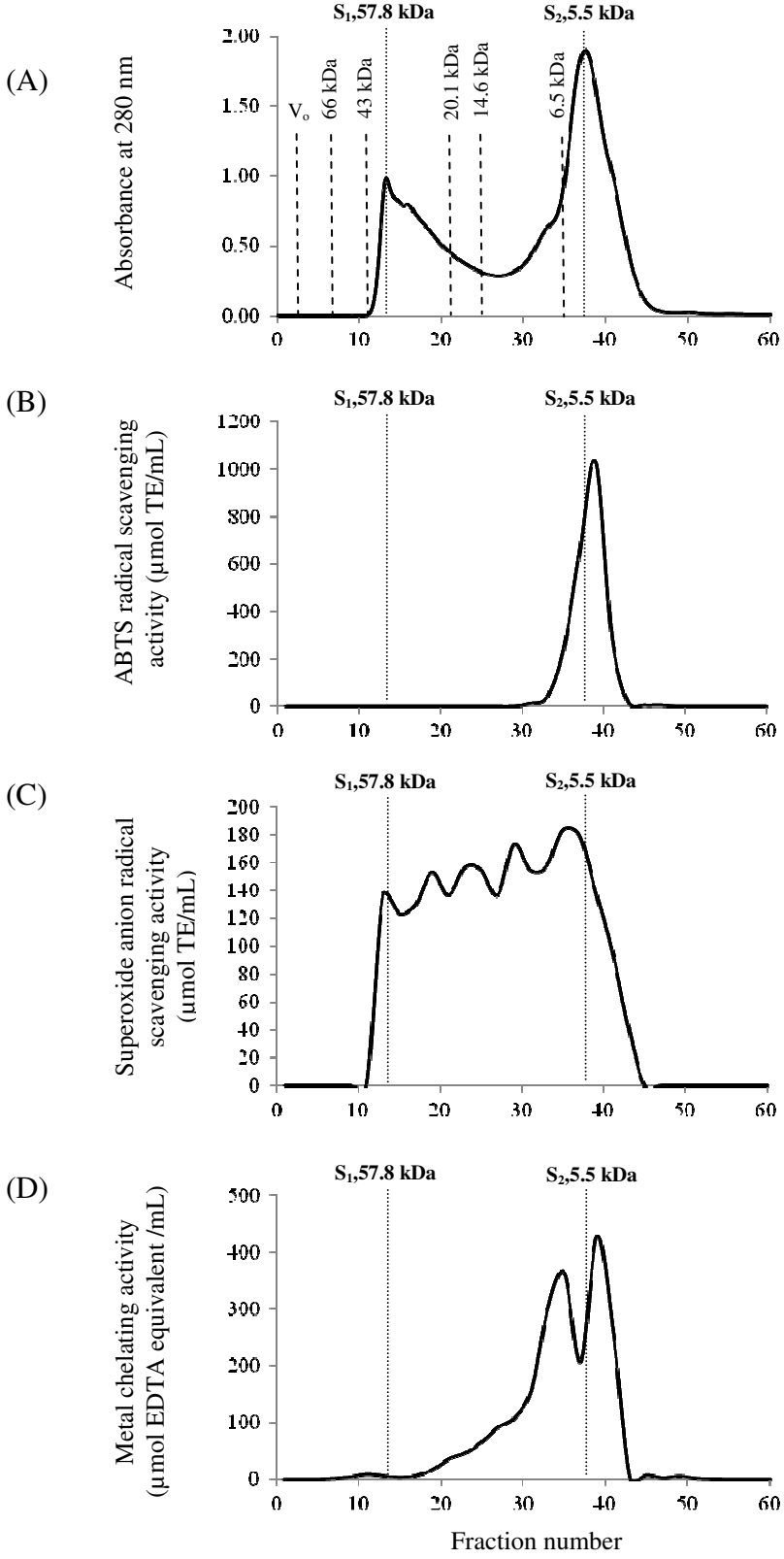


Figure 6 Elution profile by Sephadex G-75 chromatography and antioxidative axtivities of SRPH (5% DH) fractions. Absorbance at 280 nm (A), ABTS radical (B), superoxide anion radical (C) scavenging activities and metal chelating activity (D).

3.5 Conclusion

SRPH prepared by Alcalase had antioxidant activity both radical scavenging activity and metal chelating activity. The obtained hydrolysates had high solubility and interfacial properties. SRPH with 5% DH, exhibiting the highest antioxidative and functional properties, contained two major peptides with MW of 57.8 and 5.5 kDa. Thus SRPH powder from skipjack could be alternatively served as an ingredient, which can serve as the emulsifier in food emulsion and simultaneously acts as an antioxidant to prevent the lipid oxidation. Thus, the safe nutritive additive can be used in food industry.

CHAPTER 4

Isolation of antioxidative and ACE inhibitory peptides from protein hydrolysate of skipjack (*Katsuwana pelamis*) roe

4.1 Abstract

Bioactive peptides from protein hydrolysate of defatted skipjack (*Katsuwonus pelamis*) roe with 5% degree of hydrolysis (DH) prepared by Alcalase digestion were isolated and characterized. Two active fractions with ABTS radical scavenging activity (973.01 - 1497.53 µmol TE/mg sample) and chelating activity (0.05 - 0.07 µmol EE/mg sample) from consecutive purification steps including ultrafiltration, cation exchange column chromatography and reverse phase high performance liquid chromatography (RP-HPLC), were subjected to analysis of molecular mass and amino acid sequence by LC-MS/MS. Seven dominant peptides with 6-11 amino acid residues were identified as DWMKGQ, MLVFAV, MCYPAST, FVSACSVAG, LADGVAAPA, YVNDAATLLPR and DLDLRKDLYAN. These peptides were synthesized and analyzed for ACE-inhibitory activity and antioxidative activities. MLVFAV exhibited the highest ACE inhibitory activity (IC_{50} = 3.07 µM) ($p < 0.05$) with no antioxidative property, while DLDLRKDLYAN showed the highest metal chelating activity, ABTS radical and singlet oxygen scavenging activities. Therefore, Alcalase digestion could achieve skipjack roe hydrolysate with bioactivities.

4.2 Introduction

Enzymatic cleavage of fish protein has been used to produce peptides with various bioactivities such as antioxidant (Thiansilakul *et al.*, 2007a), antimicrobial (Salampessy *et al.*, 2010), anti-inflammatory (Ahn *et al.*, 2012), antihypertensive activities (Ghassem *et al.*, 2011; Li *et al.*, 2012), etc. Bioactive peptides with 3-20 amino acid residues from marine sources could be generated by various proteases (Bougatef *et al.*, 2008; Qian *et al.*, 2007; Samaranayaka *et al.*, 2010). Biological properties of these peptides are governed by amino acid

composition, sequence, size and conformation (Bougatef *et al.*, 2008). Oxidative stress generated by lipid oxidation contributes to development and progression of diseases such as diabetes, cancer, neurodegenerative and inflammatory diseases. Furthermore, oxidation of lipids in food system, particularly those containing polyunsaturated fatty acids, is a cause of nutrition loss and the release of oxidative products as well as free radicals. It also contributes to undesirable flavor, leading to the shortened shelf-life of product (Kumar *et al.*, 2012).

Hypertension or high blood pressure, a chronic disease, is one of the major risk factors for stroke, coronary heart disease (CHD), kidney dysfunction, myocardial infarction (Li *et al.*, 2012). It was estimated that 20% of the world's adult population is afflicted with hypertension and related disease (Li *et al.*, 2012). Hypertension is regulated by the catalytic activity of angiotensin I converting enzyme (ACE, EC 3. 4. 15. 1), which is classified as zinc protease (Ghassem *et al.*, 2011). ACE catalyses the conversion of angiotensin I (DRVYIHPFHL) to angiotensin II (DRVYIHPF, a vasoconstrictor) and catalyses the degradation of bradykinin (a vasodilator), resulting in high blood pressure (Ghassem *et al.*, 2011). Synthetic ACE inhibitors such as captopril, enalapril, alacepril or lisinopril are used as the first-line therapeutic approach for treatment of hypertension (Je *et al.*, 2004). However, they are reported to have certain side-effects including inflammatory response, dry cough, taste disturbance or angioneurotic edema in some patients (Wijesekara and Kim, 2010). Thus, ACE inhibitors from natural source have gained increasing attention as potential alternatives to antihypertensive drugs.

Enzymatic hydrolysis of fish protein could liberate antioxidative peptides with other biological activities such as ACE inhibitory activity (Alemán *et al.*, 2011; Harnedy and FitzGerald, 2012; Samaranayaka *et al.*, 2010). Alemán, *et al.* (2011) reported that peptide isolated from gelatin hydrolysate from inner and outer tunic of jumbo flying squid hydrolyzed by Alcalase exhibited ACE-inhibitory activity and antioxidative properties including radical scavenging activity and ferric reducing power. Protein hydrolysates from Atlantic salmon, Coho salmon, Alaska pollack, and Southern blue whiting muscle contained peptides with multifunctions, e.g. DPPH radical scavenging activity and ACE inhibitory acitivity (Nakajima *et al.*, 2009).

Roe from skipjack tuna is rich in protein and contains several amino acids, especially glutamine/glutamic acid (12.65%), leucine (8.44%) and lysine (8.30%) (Intarasirisawat *et al.*, 2011). Furthermore, protein hydrolysate of skipjack tuna roe was able to retard lipid oxidation in food model systems (Intarasirisawat *et al.*, 2014). For better understanding of bioactivities of hydrolysate from tuna roe, the peptides contributing to those activities should be examined. Nevertheless, there is scanty information about bioactive peptides from protein hydrolysate of skipjack tuna roe was reported. Thus, the aim of this study was to isolate and characterize peptides possessing ACE inhibitory activity and antioxidative properties from protein hydrolysate of skipjack tuna roe.

4.3 Materials and methods

4.3.1 Chemicals

N-Hippuryl-His-Leu hydrate (HHL), Angiotensin I converting enzyme (ACE), hippuric acid (HA), 3-(2-pyridyl)-5,6-diphenyl-1,2,4-triasine-*p*,*p'*-disulfonic acid monosodium salt hydrate (ferrozine), 2,2-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt (ABTS) and 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (Trolox) were purchased from Sigma Chemical Co. (St. Louis, MO, USA). Trifluoroacetic acid (TFA) was obtained from Acros Organics (Fair Lawn, NJ, USA). HPLC-grade acetonitrile was bought from Fisher Scientific (Nepean, ON, Canada). Hiprep SP FF (16/10) column (16 mm × 100 mm) and X Bridge^{TM} Prep C18 column (10 mm × 150 mm) were obtained from GE Healthcare (Uppsala, Sweden). All reagents were of analytical grade, except acetonitrile, which was of HPLC grade.

4.3.2 Preparation of protein hydrolysate

Frozen roes of skipjack (*Katsuwonus pelamis*) obtained from Songkhla Canning Company (Ltd), Songkhla, Thailand, were thawed and defatted as described by Intarasirisawat *et al.*(2012). Protein hydrolysate with 5% DH was prepared using Alcalase as per the method of Intarasirisawat *et al.* (2012). The obtained skipjack roe protein hydrolysate (SRPH) was lyophylized using a freeze-dryer (CoolSafe 55,

ScanLaf A/S, Lynge, Denmark), placed in polyethylene bag and kept at -20 °C until analyses.

4.3.3 Determination of bioactivities

Prior to determination of ACE inhibitory activity, sample was dissolved in 100 mM potassium phosphate buffer (pH 8.3) containing 300 mM NaCl. Sample was dissolved in distilled water for antioxidative activity determination.

4.3.3.1 Angiotensin I converting enzyme inhibitory activity

ACE inhibitory activity was measured according to the method of Cushman and Cheung (1971) with slight modifications. All reagents except HCl were freshly prepared by dissolving in 100 mM potassium phosphate buffer (pH 8.3) containing 300 mM NaCl. Fifty microliters of 5 mM HHL were added with 10 µL of sample and the mixture was then pre-incubated at 37 °C for 10 min. The reaction was initiated by adding 20 µL of ACE solution (100 mU/mL) and incubated at 37 °C for 30 min. To terminate reaction, 125 µL of 1 M HCl were added and mixed well. The mixture was filtered through 0.2 µm polyvinylidene fluoride (PVDF) disposable Iso-disc^{TM} syringe tip filter (Sigma-Aldrich, Danver, MA, USA). The filtrate was evaluated for ACE inhibitory activity using an Acquity UPLC BEH C18 column (50 mm × 2.1 mm, id, 1.7 µm particle size, Waters). The peak area of HA at absorbance 228 nm was calculated for the amount of HA. HA at varying concentrations (0.7-42.0 µg/mL) was used as standard. Captopril and 100 mM phosphate buffer (pH 8.3) containing 300 mM NaCl were used as positive and negative control, respectively. Sample concentrations vs % inhibition were plotted as nonlinear regression. IC_{50} was calculated and expressed as µg sample/mL or µM.

4.3.3.2 ABTS radical scavenging activity

ABTS radical scavenging activity was measured as per the method of Thiansilakul *et al.* (2007a). The stock solutions included 14.8 mM ABTS solution and 5.2 mM potassium persulfate solution. The working solution was prepared by mixing two stock solutions in equal quantities and allowing them to react for 12 h at room

temperature in dark. The solution was then diluted by mixing 1 mL of ABTS solution with 50 mL of methanol in order to obtain an absorbance of 1.1 ± 0.02 units at 734 nm using a UV-160 spectrophotometer (Shimadzu, Kyoto, Japan). Fresh ABTS solution was prepared daily. Sample (150 µL) with different solid concentrations (5, 10 and 20 mg/mL) was mixed with 2850 µL of ABTS solution and the mixture was left at room temperature for 2 h in dark. The absorbance at 734 nm was measured. A standard curve of Trolox ranging from 50 to 600 µM was prepared. The activity was expressed as µmol Trolox equivalents (TE)/mg sample.

4.3.3.3 Hydrogen peroxide scavenging activity

Hydrogen peroxide scavenging activity was assayed according to the method of Kittiphattanabawon *et al.* (2012). Sample (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℃. 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)/mg sample.

4.3.3.4 Singlet oxygen scavenging activity

Singlet oxygen scavenging activity was determined as described by Kittiphattanabawon *et al.* (2012). The chemical solutions and sample were prepared in 45 mM sodium phosphate buffer (pH 7.4). The reaction mixture consisted of 0.4 mL of sample, 0.5 mL of 200 µM *N*,*N*-dimethyl *p*-nitrosoaniline (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, and 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 blanks were 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)/mg sample.

4.3.3.5 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 0-3 µM was used as standard. Metal chelating activity was expressed as µmol EDTA equivalent (EE)/mg sample.

4.3.4 Isolation of bioactive peptides

4.3.4.1 Ultrafiltration

SRPH (7.55 g) was suspended in 188.77 mL of 10 mM ammonium acetate, pH 4.0 and filtered through an Amicon stirred ultrafiltration cell (Model 8200, Merck Millipore, Bedford, MA, USA) using a 3 kDa molecular weight cut-off (MWCO) membrane (Millipore Corporation, Bedford, MA, USA). The retentate (>3 kDa) and permeate (<3 kDa) were collected. ACE inhibitory activity and antioxidative activities (ABTS radical scavenging activity and metal chelating activity) of both retentate and permeate were determined.

4.3.4.2 Cation exchange chromatography

The permeate obtained from ultrafiltration (2.5 mL) was loaded onto the Hiprep SP FF (16/10) column (16 mm × 100 mm, Amersham Biosciences, Hillerød, Denmark), connected with an AKTA fast performance liquid chromatography (FPLC) system (Amersham Biosciences, Montréal, QC, Canada). Sample (100 mg/mL) was eluted with 10 mM ammonium acetate, pH 4.0 (buffer A) for 2 column volumes (CV), gradient with 0.5 M ammonium carbonate, pH 8.8 (buffer B) from 0-5% for 10 CV, then increased to 100% buffer B for 0.5 CV and held for 2 CV. Flow rate was maintained at 5 mL/min. Fractions (5 mL) were collected and then concentrated by lyophilization. The lyophilized samples were determined for ABTS radical scavenging activity, metal chelating activity and ACE inhibitory activity. Fraction possessing high ACE inhibitory activity and antioxidative

properties was chosen for further purification using the reversed phase HPLC chromatography.

4.3.4.3 Preparative reversed phase chromatography

The selected fraction obtained from cation exchange chromatography was further fractionated by a 10 mm × 150 mm i.d., 5 μm, XBridge^{TM} Prep C_{18} column (Waters Inc., Milford, MA, USA) attached to a Waters 600 HPLC system. Sample (900 μL) was automatically injected by a Waters 2707 autosampler, eluted by acetonitrile containing 0.1% trifluoroacetic acid (mobile phase B) from 0 to 40% at a flow rate of 5 mL/min. The absorbance at 220 nm and 280 nm was monitored. Fractions (15 mL) were collected every 3 min by a Waters Fraction Collector III controlled by Empower version 2 software. The obtained fractions were concentrated using a vacuum-rotary evaporator at 35 °C and determined for ACE inhibitory activity, ABTS radical scavenging activity and chelating activity. Fractions showing high ACE inhibitory activity (Fraction C-IV and C-VII) were subjected to identification of peptide sequence using LC-MS/MS.

4.3.5 LC-MS/MS

Identification of the potent peptides in the fraction from reverse phase chromatography was carried out by liquid chromatography-tandem mass spectrometry (LC–MS/MS). The analysis was carried out in Waters (Micromass) Q-TOF Premier (Milford, MA, USA) and sample was separated by Waters Atlantis dC18 (75 μm × 150 mm, 3 μm) UPLC column (Milford, MA, USA). The dual solvent system was composed of solvent A (0.1% formic acid in optima LC/MS grade water) and solvent B (0.1% formic acid in optima grade acetonitrile). Samples were prepared in solvent A; then 5 μL of sample was injected to the 5 μm trapping column. Sample was trapped for 2 min at a flow rate of 10 μL/min using 99% solvent A, followed by a gradient from 99% to 90% solvent A over 5 min, to 70% solvent A over 30 min, to 60% solvent A over 3 min and 5% solvent A over 1 min at a constant flow rate of 0.350 μL/min. Then the flow rate was increased to 0.500 μL/min, held at 5% solvent A for 2 min, increased it to 98% solvent A over 1 min, held for another 27 min, and

then decreased to 0.350 µL/min over 1 min. Ionisation was performed using electrospray ionisation technique (ESI) by nanoLockspray ionisation source in a positive ion mode (capillary voltage at 3.80 kV and the source temperature at 100 °C). Peptide mass was determined through Q-TOF analyser operated in a positive ion MS/MS mode. A MS/MS full-scan was performed for each sample with an acquisition *m*/*z* range of 0–1000 Da. The peptides were then characterized using Peaks Viewer 4.5 software (Bioinformatics Solutions Inc., Waterloo, ON Canada) and the peptide sequences were identified from respective mono-isotopic mass. Peptide distributions were obtained (LC-MS/MS chromatographs). Dominant peptide of each peak was identified for mass, sequence and blasted with NCBI database (Non-redundant protein sequences (nr) and algorithm (protein-protein blast). Peptides characterized from major roe proteins (vitellogenin and membrane protein) were chosen for synthesis by Genscript (Piscataway, NJ, USA).

4.3.6 Synthesis of peptides and determination of bioactivities

Synthesis of peptides identified by LC-MS/MS was performed by a solid-phase technique (GenScript Corporation, Piscataway, NJ, USA). The purity of the synthesized peptides was greater than 95% as determined by HPLC analysis. All peptides were determined for ACE inhibitory activity and antioxidative activities as described previously.

4.3.7 Statistical analysis

All experiments were run in triplicate. All analyses were conducted in triplicate, except for ACE inhibitory activity, which was performed in duplicate. Statistical analysis was performed using one-way analysis of variance (ANOVA). Mean comparison was carried out using Duncan's multiple range (Steel and Torrie, 1980). Analysis was performed using the SPSS package (SPSS for windows, SPSS Inc., Chicago, IL, USA).

4.4 Results and discussions

4.4.1 Purification of bioactive peptides from SRPH

SRPH (5% DH) showed higher antioxidative activities, when compared with other SRPH with higher DHs (10, 20, 30, 40 and 50%) (Intarasirisawat *et al.*, 2012). To isolate the active peptides, ultrafiltration was firstly used. IC_{50} of ACE inhibitory activity of SRPH was 2,497.31 µg sample/mL. ABTS radical scavenging activity and chelating activity of SRPH were 393.31 µmol TE/mg sample and 6.98 µmol EE/mg sample, respectively. After ultrafiltration, ACE inhibitory activity of permeate (IC_{50} = 761.82 µg sample/mL) was better than that of retentate (IC_{50} = 2,311.41 µg sample/mL). ABTS radical scavenging activity of permeate (444.00 µmol TE/mg sample) had slightly higher than that of retentate (165.27 µmol TE/mg sample). Nevertheless, the retentate (12.08 µmol EE/mg sample) showed the higher chelating activity than that of permeate (5.32 µmol EE/mg sample). The results suggested that peptides with MW less than 3 kDa exhibited superior ACE inhibitiory activity and ABTS radical scavenging activity to those found in retentate. The permeate was further purified by cation exchange column chromatography. Nine fractions (A-I) were obtained and determined for ACE inhibitory activity, ABTS radical scavenging activity and metal chelating activity (Table 10). Apparently, cation exchange chromatogram (Figure 11) indicated that most abundant peptides in permeate more likely had less positive charge as evidenced by high absorbance intensity of both 280 and 220 nm particularly in the unbounded fractions A-D. Highly positive charged peptides were eluted (fraction E-I) with increasing concentrations of ammonium carbonate. IC_{50} of ACE inhibitory activity, ABTS radical scavenging activity and metal chelating activity of the obtained fractions were varied. Peptides with antioxidative properties were mainly found in the first four fractions (A-D), particularly those having metal chelating activity. The lowest IC_{50} of ACE inhibitory was obtained for fraction B with the highest ABTS radical scavenging activity ($p < 0.05$). Nevertheless, fraction C exhibiting high ACE inhibitory activity (IC_{50} = 214.69 µg sample/mL) and ABTS radical scavenging activity (640.85 µmol TE/mg sample) had the higher yield than fraction B by 1.3 fold.

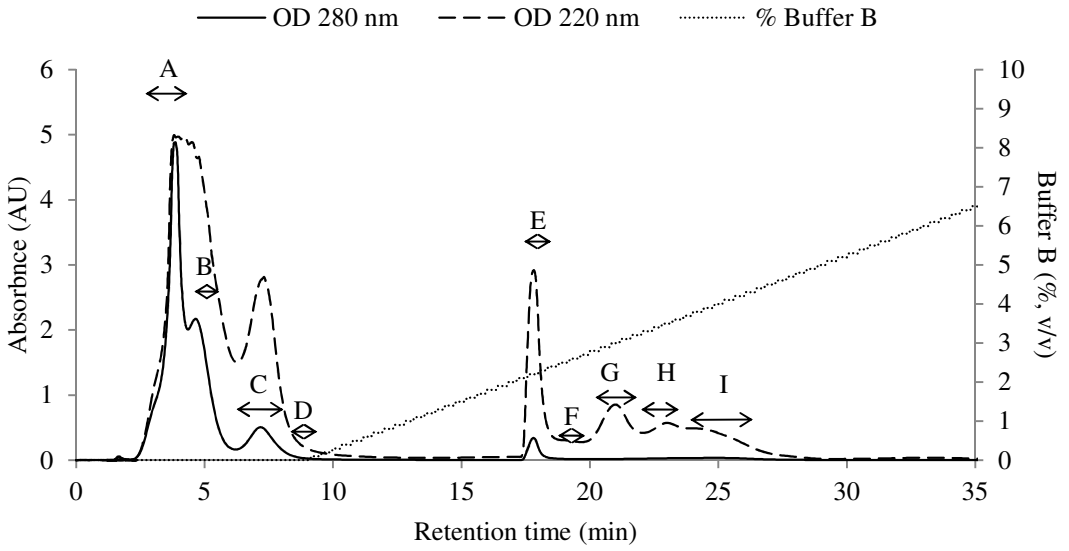


Figure 1 Cation exchange chromatogram of SRPH having 5% DH. ↔represent the pooled fractions

Table 1 Bioactivities of fractions obtained from cation exchange chromatography

Fractions	IC_{50} of ACE inhibitory activity (µg sample/mL)[†]	ABTS scavenging activity (µmol TE/mg sample)[‡]	Chelating activity (µmol EE/mg sample)[‡]
A	6,341.25[‡]	246.79±12.19^{E}	17.07±0.29^{A}
B	191.81	829.42±21.50^{A}	2.22±0.11^{B}
C	214.69	640.85±9.16^{B}	0.84±0.13^{C}
D	220,153.78	290.89±18.89^{D}	0.28±0.12^{D}
E	8,497.76	164.25±14.14^{F}	ND
F	10,311.53	100.54±8.60^{G}	ND
G	350.81	235.59±29.23^{E}	ND
H	372.91	381.30±29.14^{C}	0.14±0.09^{D}
I	248.70	102.17±12.56^{G}	ND

[†]IC_{50} value was defined as the concentration of inhibitor required to inhibit 50% of ACE activity and obtained from duplicate determinations.

[‡]Values are mean ± SD (n = 3).

ND: not determined.

Different superscripts within the same column indicate the significant differences ($p < 0.05$).

The high ACE inhibitory activity of fractions B and C might presumably relate with the ability to chelate metal in zinc-ligand of ACE (Kang *et al.*, 2003; Qian *et al.*, 2007). High antioxidative activity was plausibly due to the ability in donating hydrogen of peptides (Qian *et al.*, 2007). Therefore SRPH containing peptides derived from phosvitin (a phosphate containing protein) could inhibit ACE via metal chelation. In addition to inhibit ACE activity, the availability of positive charge of guanidine group from arginine (R) in peptide also contributes to inhibition via binding with carboxylate active site of ACE (Kang *et al.*, 2003). Kang *et al.* (2003) reported that the presence of hydrophobic amino acid residue at C-terminal can inactivate ACE activity by accommodating the alteration of hydrophobic catalytic site of ACE. The active fraction C which had higher recovery yield (7.52% of total SRPH), compared to fraction B (3.30% of total SRPH), was then further fractionated by using a reverse phase chromatography, eight fractions were obtained and evaluated for ACE inhibitory activity and antioxidative activities as shown in Figure 12 and Table 11.

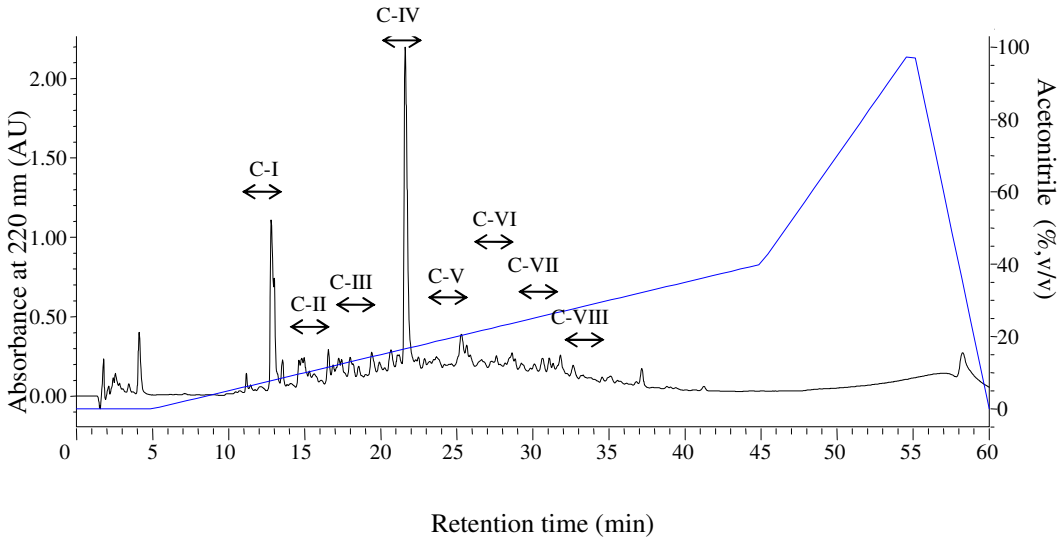


Figure 2 Reverse phase HPLC chromatogram of fraction C obtained from cation exchange chromatography. ↔represent the pooled fractions.

With increasing acetonitrile concentration, higher hydrophobic residues were eluted. Among all fractions, the highest ACE-inhibitory activity could be observed in fraction C-VII (IC_{50} = 1.92 µg sample/mL), followed by fraction C-IV

(IC_{50} = 4.31 µg sample/mL). The highest ACE-inhibitory activity of fraction C-VII might be due to high hydrophobicity which made the peptide having higher affinity to inhibit ACE activity (Jung *et al.*, 2006b). Fraction C-VII also showed the highest ABTS radical scavenging activity. No difference in metal chelating activity was observed for both fractions, C-IV and C-VII ($p > 0.05$). Fraction C-IV and C-VII with high ACE inhibitory activity and antioxidative activity were selected for identification of molecular mass and amino acid sequence using LC-MS/MS.

Table 2 Bioactivities of fractions obtained from reverse phase high performance liquid chromatography

Fractions	IC_{50} of ACE inhibitory activity (µg sample/mL)[†]	ABTS scavenging activity (µmol TE/mg sample) [‡]	Chelating activity (µmol EE /mg sample) [‡]
C-I	1870.77	181.71 ± 1.19^{G}	0.27 ± 0.01^{A}
C-II	1623.22	279.52 ± 0.28^{F}	0.14 ± 0.01^{B}
C-III	120.77	204.29 ± 17.86^{G}	0.05 ± 0.05^{C}
C-IV	4.31	973.01 ± 63.27^{C}	0.07 ± 0.01^{C}
C-V	146.50	856.94 ± 45.64^{D}	ND
C-VI	8.09	615.33 ± 18.48^{E}	ND
C-VII	1.92	1497.53 ± 19.34^{A}	0.04 ± 0.01^{C}
C-VIII	939.95	1137.78 ± 6.60^{B}	0.03 ± 0.01^{C}

[†]IC_{50} value was defined as the concentration of inhibitor required to inhibit 50% of ACE activity and obtained from duplicate determinations.

[‡]Values are mean ± SD (n = 3).

Different superscripts within the same column indicate the significant differences ($p < 0.05$).

4.4.2 Identification of peptides

From Figure 13A, there were four major peaks in fraction C-IV. However, only peak eluted at 20.09 min contained the potential ACE peptide (FVSACSVAG) as determined by mass distribution (Figure 13B) and amino acid sequencing (Figure 13C). For fraction C-VII (Figure 14), six potential peptides with ACE inhibitory activity were identified to include DWMKGQ (blast score = 53 (25.7 bits)), MLVFAV (blast score = 47 (23.1 bits)), MCYPAST (blast score = 51 (24.8 bits)), LADGVAAPA (blast score = 60 (28.6 bits)), YVNDAATLLPR (blast score = 77 (35.8 bits)) and DLDLRKDLYAN (blast score = 78 (36.3 bits)). Based on blast score, it was suggested that those peptides were more likely derived from egg protein. All seven potential ACE inhibitory peptides were then synthesized and determined for ACE inhibitory activity (Table 12). Varying inhibitory activity of these peptides might be attributed to different amino acid compositions, hydrophobicity, etc (Jung *et al.*, 2006b; Wijesekara and Kim, 2010). Among seven synthetic peptides, MLVFAV (IC_{50} = 3.07 μM, 678 Da) had the strongest ACE inhibitory activity, followed by FVSACSVAG (IC_{50} = 3.49 μM, 839 Da). This was possibly due to the availability of hydrophobic amino acid residues (leucine (L), valine (V) and alanine (A)), which preferably bind with catalytic sites of ACE (S'_1, a penultimate position) (Jung *et al.*, 2006b). Moreover, the presence of phenylalanine (F) at C-terminus of this peptide was reported to be more favorable for the ACE inhibition (Jung *et al.*, 2006b). Wijesekara *et al.* (2010) suggested that peptides containing tryptophan (W), tyrosine (Y), proline (P) or phenylalanine (F) at C-terminal and branched-chain aliphatic amino acids at N-terminal more likely act as the strong competitive ACE inhibitor. Furthermore, the presence of hydrophobic amino acid residues at either terminus in peptide plausibly contributed to ACE inhibitory activity (Wijesekara and Kim, 2010).

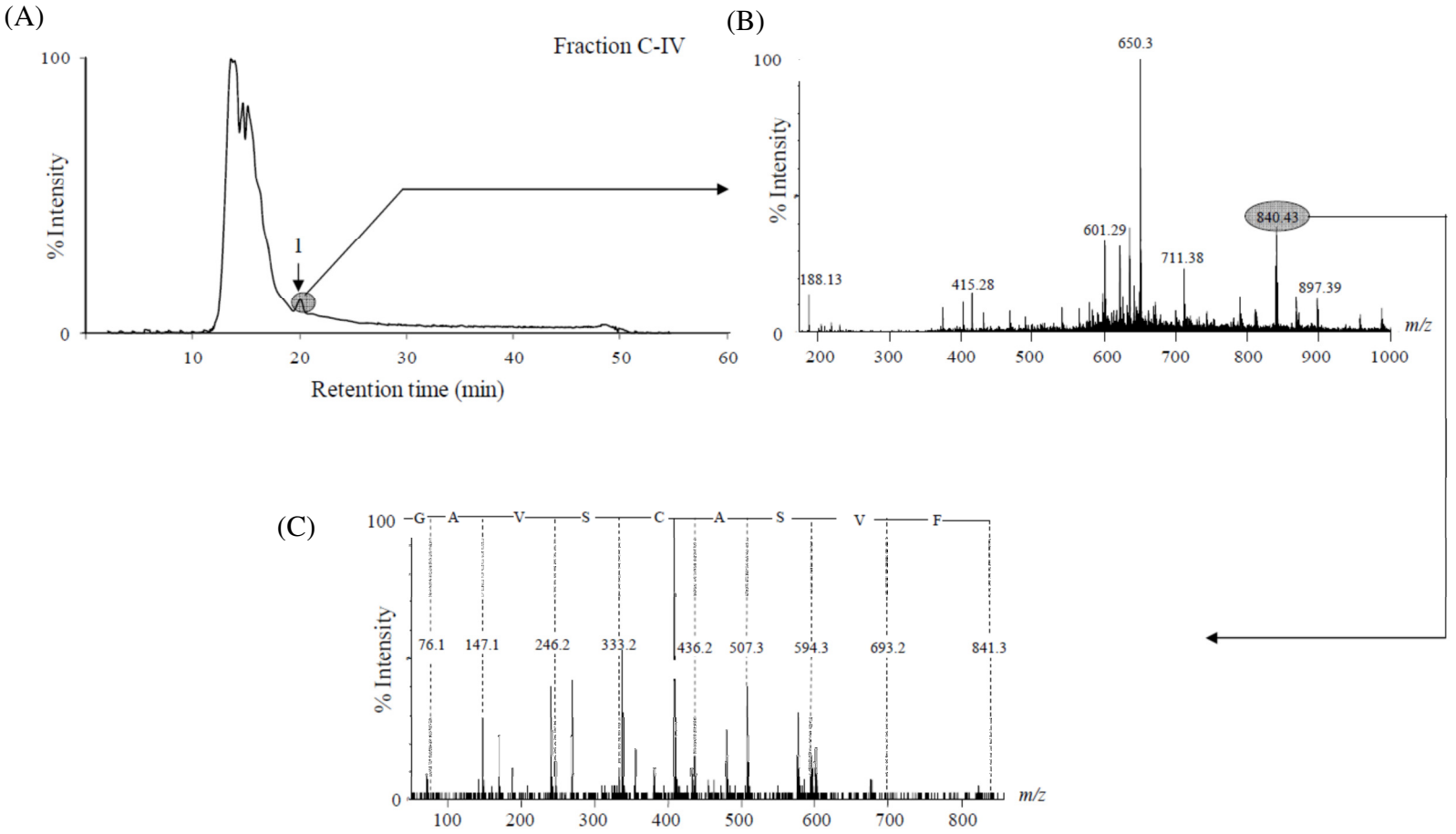


Figure 3 De novo sequencing of potential ACE inhibitory peptides from the active RP-HPLC fraction C-IV (FVSACSVAG). (A) LC-MS chromatogram of fraction C-IV: (B) full MS scan: (C) MS/MS spectrum of the ion 840.4 *m/z*.

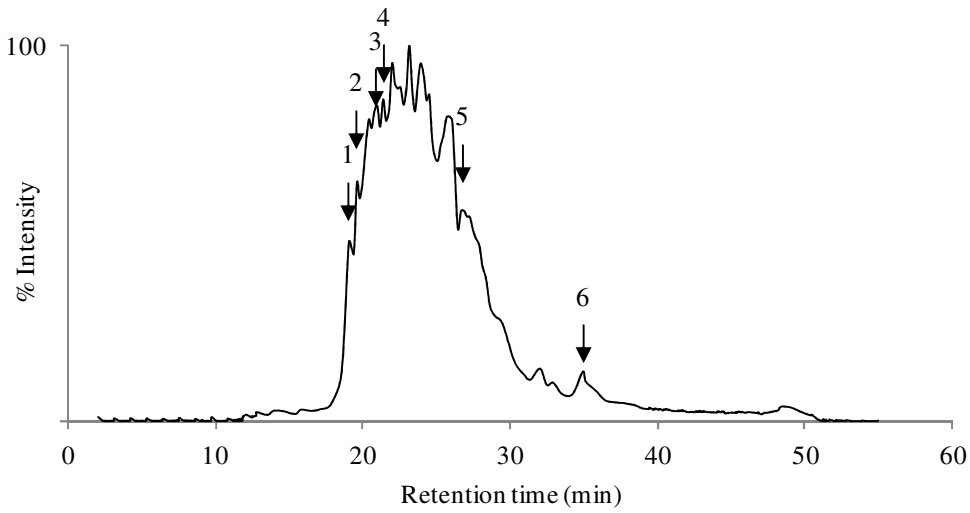


Figure 4 LC-MS chromatogram of potential ACE inhibitory peptides from the active RP-HPLC fraction C-VII. Arrows indicate the fraction with ACE inhibitory activity

Numerous ACE inhibitory peptides with varying chain lengths from fish protein hydrolysates were reported. ACE inhibitory ability of MLVFAV isolated from SRPH was higher than PEAAELMMEVDP (IC_{50} = 21.6 µM, 1,581 Da) from tuna dark muscle (Qian *et al.*, 2007), GDLGKTTTVSNWSPPKYKDTP (IC_{50} = 11.28 µM, 2,482 Da) from tuna frame (Lee *et al.*, 2010), AHLL (IC_{50} = 40.3 µM, 452 Da) from Loach meat (Li *et al.*, 2012) but lower than VPAAPPK (IC_{50} = 0.45 µM, 791 Da) and NGTWFEPP (IC_{50} = 0.63 µM, 1085 Da) from Haruan myofibrilar protein (Ghassem *et al.*, 2011) and CF (IC_{50} = 1.96 µM, 267 Da) and FE (IC_{50} = 1.45 µM, 294 Da) from shark meat (Wu *et al.*, 2008). Nevertheless, ACE inhibitory activity of MLVFAV was lower than that of commercial synthetic ACE inhibitor such as captopril (IC_{50} = 0.1 µM) (Je *et al.*, 2004).

Table 3 Bioactivities of the selected synthetic peptides

Sequence NO.	Sequences[†]	Mass (Da)	IC_{50} of ACE inhibitory activity (μM)[‡]	Metal chelating activity*		ABTS scavenging activity*		Hydrogen peroxide scavenging activity*		Singlet oxygen scavenging activity*	
				μmol EE/mg sample	mol EE/mol sample	μmol TE/mg sample	mol TE/mol sample	μmol TE/mg sample	mol TE/mol sample	μmol TE/mg sample	mol TE/mol sample
Fraction C-IV											
1	FVSACSVAG	839	3.49	ND	ND	1175.15±44.94^{C}	985.95±37.71^{C}	2.88±0.02^{C}	2.42±0.01^{C}	0.88±0.09^{B}	0.74±0.07^{B}
Fraction C-VII											
1	DWMKGQ	763	422.92	ND	ND	15.85±0.46^{D}	12.09±0.35^{D}	ND	ND	0.01±0.00^{C}	0.01±0.00^{C}
2	LADGVAAPA	854	317.75	0.01±0.00^{B}	0.01±0.00^{B}	1.44±0.31^{D}	1.23±0.27^{D}	7.09±0.02^{A}	6.06±0.01^{A}	ND	ND
3	YVNDAATLLPR	1231	105.85	ND	ND	18.67±3.38^{D}	22.99±4.16^{D}	ND	ND	ND	ND
4	DLDLRKDLYAN	1334	67.43	28.21±0.32^{A}	37.64±0.42^{A}	4853.81±0.08^{A}	6474.99±0.11^{A}	4.11±0.03^{B}	5.49±0.04^{B}	3.41±0.00^{A}	4.54±0.00^{A}
5	MCYPAST	771	58.73	ND	ND	1366.03±36.76^{B}	1053.21±28.34^{B}	ND	ND	ND	ND
6	MLVFAV	678	3.07	ND	ND	ND	ND	ND	ND	ND	ND

[†]Peptide sequences are characterized by Peak viewer 4.5 software.

[‡]IC_{50} value was defined as the concentration of inhibitor required to inhibit 50% of ACE activity and obtained from duplicate determinations.

*Values are mean ± SD (n = 3).

Different superscripts within the same column indicate the significant differences ($p < 0.05$).

4.4.3 Antioxidative properties of synthetic peptides

Antioxidative properties including metal chelating activity, ABTS radical, hydrogen peroxide and singlet oxygen scavenging activities are shown in Table 3. No metal chelating activity was detected in all peptides, except DLDLRKDLYAN. When comparing with original SRPH (6.98 µmol EE/mg sample), DLDLRKDLYAN (28.21 µmol EE/mg sample) had higher chelating activity.

All peptides exhibited ABTS radical scavenging activity, except MLVFAV. The highest ABTS radical scavenging activity was observed for DLDLRKDLYAN (4853.81 µmol TE/mg sample) ($p < 0.05$) which is higher than that of original SRPH (393.31 µmol TE/mg sample) by 12-fold. No ABTS radical scavenging activity was detected for MLVFAV, which had the high content of hydrophobic amino acid residues (L, V and A). Generally peptides containing hydrophobic amino acids, such as P, L, A, W and F, are believed to possess high antioxidant activity (Jung *et al.*, 2006b). Low ABTS radical scavenging activity of MLVFAV might be owing to the improper configuration of peptide for scavenging ABTS radicals.

Capacity of scavenging of hydrogen peroxide (H_2O_2) and singlet oxygen ($^1O_2$) of seven synthetic peptides is presented in Table 12. Hydrogen peroxide and singlet oxygen as reactive oxygen species (ROS) can cause oxidative stress and damage biomolecule in the cell, leading to cell death and serious chronic diseases (Suh *et al.*, 2011). Hydrogen peroxide, a weak oxidizing agent, is implicated indirectly in lipid oxidation. Hydrogen peroxide is a reactive non radical, which can permeate biological membranes and be converted to more reactive species as hydroxyl radical and singlet oxygen (Choe and Min, 2005). Among all peptides, four peptides exhibited no hydrogen peroxide scavenging activity. LADGVAAPA showed the highest hydrogen scavenging activity (7.09 µmol TE/mg sample) ($p < 0.05$), compared with the other synthetic peptides. The hydrogen peroxide scavenging activity of three synthetic peptides including FVSACSVAG, LADGVAAPA and DLDLRKDLYAN might be attributed to the availability of P, A, L, F and Y in their sequence. Byun *et al.* (2009) reported that peptides containing above amino acids

contributes to strong scavenging effect of ROS. Guo *et al* (2009) reported that the presence of Y residue at its C-terminus was associated with strong hydrogen peroxide scavenging activity. For the other peptides with no quenching activity toward hydrogen peroxide, this might be related with the unsuitable amino acid arrangement and peptide conformation (Elias *et al.*, 2008).

The highest singlet oxygen scavenging activity was observed for DLDLRKDLYAN (3.41 μmol TE/mg sample) ($p < 0.05$), followed by FVSACSVAG (0.88 μmol TE/mg sample). Amino acids are recognized as potential singlet oxygen scavenger, particularly P, A, L, F, W and Y (Byun *et al.*, 2009). The aromatic amino acids (Y and F) could make active oxygen stable through direct electron transfer (Qian *et al.*, 2007). Additionally, the presence of histidine (H) and methionine (M) also enhanced the scavenging activities of peptides (Rajapakse *et al.*, 2005b). 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. Since 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). The results revealed that the 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.

4.5 Conclusion

Alcalase-hydrolyzed protein from skipjack roe (5% DH), a by-product from tuna canning industry, contained peptides possessing ACE inhibitory activity along with antioxidative activity. MLVFAV exhibited the highest inhibitory activity toward ACE, while DLDLRKDLYAN showed the highest metal chelating activity, ABTS radical and singlet oxygen scavenging activities. Therefore, SRPH could be

potentially served as a functional food ingredient with pharmaceutical function against hypertension symptom and diseases associated with radicals.

CHAPTER 5

Effects of skipjack roe protein hydrolysate on properties and oxidative stability of fish emulsion sausage

5.1 Abstract

Effects of skipjack roe protein hydrolysate (SRPH) at various levels (0-3%) on properties and oxidative stability of emulsion sausage from broadhead catfish (*Clarias macrocephalus*) fortified with skipjack tuna roe lipids were investigated. The addition of SRPH increased hardness, cohesiveness and resilience of sausage ($p < 0.05$). Finer fat globules were visualized in the sample added with SRPH at higher amounts. Nevertheless, the incorporation of SRPH at all levels had no impact on likeness of sausages. SRPH was shown to retard lipid oxidation of sausage during extended storage of 12 days as evidenced by the lower peroxide value (PV) and thiobarbituric acid substances (TBARS), in comparison with the control. After 12 days, the sausage with 3% SRPH had the retained docosahexaenoic acid (DHA) and eicosapentaenoic acid (EPA), accounting more than 80%. Addition of SRPH had no effect on the organoleptic properties but could prevent the development of rancidity. Nevertheless, it showed no pronounced impact on microbial growth. SRPH could therefore be used as a natural antioxidative emulsifier in cooked fish emulsion sausage.

5.2 Introduction

Emulsion sausage is commercially prepared by poultry, pork or beef emulsified with backfat from several sources. During comminution, bundles of fibres, myofibrils and filaments are disrupted and the size of fatty tissues is continuously reduced. In the presence of salt, myofibrillar proteins become soluble and migrate to the fat globule surface, concentrate and form protein matrix at the fat/water interface (Heinz, 1991; Youssef and Barbut, 2010). During comminuting, the temperature is usually controlled to prevent the mechanical overheating, which could lower the emulsifying property of proteins. This is associated with the migration of

un-emulsified fat to the product surface (Heinz, 1991; Liu *et al.*, 2000). Collapse of emulsion in sausage negatively affects the texture, mouth feel and overall acceptance (Youssef and Barbut, 2009). As a result, the application of emulsifier is essential to improve the stability of emulsion product.

Recently, meat or fishery products fortified with n-3 fatty acids have considerable market growth due to health concern. Recommended daily intake of total n-3 polyunsaturated fatty acids (PUFAs) around 3–5.5 g has been made by British Nutrition Foundation (1992) but should not exceed 3.0 g/day in the form of fish oil, food and dietary supplements sources (FDA, 2000). The substitution of pork or beef backfat, mainly comprising saturated fatty acids, with fish oil possessing a high amount of PUFAs particularly DHA and EPA can enhance the nutrition quality of meat product. However, those PUFAs are susceptible to oxidation, in which undesirable off-odor can be developed. Emulsion sausages prepared from African walking catfish (*Clarias gariepinus*) and rohu (*Labeo rohita*) exhibited the lower oxidative stability when increasing amount of refined tuna oil was incorporated (Panpipat and Yongsawatdigul, 2008). Cáceres *et al.* (2008) reported that conventional and low-fat cooked sausages added with 60 g/kg fish oil were prepared with sensorial acceptability. Although oils enriched with EPA and DHA are of nutritive value, the use of oil at high proportion commonly generates product with soft texture and the resulting product is susceptible to oxidation (Cáceres *et al.*, 2008). Therefore the addition of antioxidant is required to prevent lipid oxidation. Previous *in vitro* study indicated that SRPH having 5% degree of hydrolysis (DH) prepared by Alcalase had good emulsifying properties and antioxidative activities, both radical scavenging activities and metal chelating activity (Intarasirisawat *et al.*, 2012). Additionally, different fish roe hydrolysate have been known to have antioxidative acitivities. DPPH radical scavenging activity and reducing power were found for protein hydrolysate from roe of channa (*Channa striatus*), rohu (*Labeo rohita*) (Galla *et al.*, 2012) and herring (*Clupea harengus*) (Sathivel *et al.*, 2003). Therefore, SRPH could be used as antioxidative emulsifier in oil-water-emulsion meat system. Nevertheless, there is a little information regarding the use of fish protein hydrolysate in fish emulsion sausage supplemented with fish oil containing high PUFA.

Therefore, the aim of this study was to determine the effect of skipjack roe hydrolysate on properties and oxidative stability of broadhead catfish emulsion sausage fortified with tuna roe lipids during the refrigerated storage.

5.3 Materials and methods

5.3.1 Chemicals

Cumene hydroperoxide and osmium tetraoxide were purchased from Sigma Chemical Co. (St. Louis, MO, USA). Trichloroacetic acid and glutaraldehyde were obtained from Merck (Damstadt, Germany). Thiobarbituric acid, ammonium thiocyanate and ferrous chloride were purchased from Fluka Chemical Co. (Buchs, Switzerland).

5.3.2 Extraction of tuna roe lipids

Lipids from tuna roe were extracted following the Bligh and Dyer method (1959) with silght modification. Skipjack roe (75 g) was homogenized with 150 mL of the cold chloroform-methanol mixture (1:1, v/v) for 1 min. The homogenate was added with 75 mL of chloroform and homogenized for another 30 s. The mixture was filtered through a Whatman No. 4 filter paper (Whatman International Ltd., Maidstone, UK.). The filtrate was transferred to a separating funnel and allowed to stand at 4 °C overnight. The bottom phase (chloroform phase) was drained off into Erlenmeyer flask containing sodium sulfate anhydrous (1-2 g). The mixture was shaken thoroughly to remove the residual water. Lipid in chloroform was decanted into a rounded-bottom flask through a filter paper (Whatman No. 4). The chloroform was evaporated at 25 °C using a rotary evaporator (Rotavapor, model R-14, Buchi, Tokyo, Japan). Lipids obtained were transferred into the amber bottle, flushed with N_2 gas, closed tightly and stored at -20°C until use.

5.3.3 Preparation of SRPH

SRPH with 5% DH, the percent ratio of the number of peptide bonds cleaved to the total number of peptide bonds in the substrate, was prepared as per the method of Intarasirisawat *et al.* (2012). The defatted sample (2.5 g dry matter) was

suspended in 95 mL of distilled water and pre-incubated at 50 °C for 20 min prior to enzymatic hydrolysis. The hydrolysis reaction was initiated by the addition of Alcalase and the hydrolysis was taken at 50°C, pH 8.0 for 1h. The mixture was then placed into a water bath at 85 °C for 15 min to terminate the enzymatic reaction. Hydrolysate was lyophilized using a freeze-drier (Model Duratop™ lP/Dura Dry™ lP, FTS® System, Inc., Stone Ridge, NY, USA).

5.3.4 Preparation of fish sausage

Broadhead catfish (*Clarias macrocephalus*) with the size of 1.0-1.2 kg/fish were purchased from a market in Hat Yai, Songkhla, Thailand. Fish were placed in ice using a fish/ice ratio of 1:2 and transported to the Department of Food Technology, Prince of Songkla University, within 30 min. Fish were manually decapitated, eviscerated and washed with ice water (4°C). Bone and skin were removed. Fish fillets were then minced using a meat grinder (MX-T2G National, Tokyo, Japan). Emulsion sausage was prepared following the method of Panpipat and Yongsawatdigul (2008) with a slight modification. Fish mince (60 g) was mixed with 20 g/kg NaCl at 4 °C for 5 min. Subsequently, 20 g of the mixture between sunflower oil, containing 200 mg/L vitamin E (Morakot Industries PCL, Samutprakan, Thailand) and tuna roe lipids (7:3, v/v) were added. Other ingredients including 2 g/kg tripolyphosphate, 24 g/kg tapioca starch and 10 g/kg sugar were added. SRPH was incorporated at varying concentrations (0.5, 1 and 3 g/100 g). Moisture content of all samples was adjusted to 75%. The mixture was ground for 3 min. The paste was stuffed into a 2.2 cm diameter cellophane casing. Both ends were sealed tightly. The samples were pre-incubated at 55 °C for 40 min prior to cooking at 80 °C for 15 min. The samples were placed in a polyethylene bag, sealed and kept at 4 °C.

5.3.5 Study on microstructure of fish sausage containing SRPH at different levels

The microstructure of the sausages added with SRPH at different levels was determined using a scanning electron microscope (SEM). Samples with a thickness of 2-3 mm were fixed with 25% (v/v) glutaraldehyde in 0.2 M phosphate

buffer (pH 7.2). Fixed samples were washed with 0.1 M phosphate buffer (pH 7.2) for 10 min and then post-fixed in 0.2 M phosphate buffer (pH 7.2) containing 1% (w/v) osmium tetraoxide for 1 h. The fixed samples were then rinsed in 0.1 M phosphate buffer for 10 min and rinsed with distilled water for 10 min before being dehydrated in ethanol with serial concentrations of 50, 70, 80, 90 and 100%. Dried samples were mounted on a bronze stub and sputter-coated with gold (Sputter coater SPI-Module, West Chester, PA, USA). The specimens were observed with a scanning electron microscope (JEOL JSM-5800 LV, Tokyo, Japan) at an acceleration voltage of 10 kV.

5.3.6 Effect of SRPH on properties of emulsion sausage during refrigerated storage

Sausages fortified with tuna roe lipids and added with SRPH at different levels were monitored for chemical, textural and microbiological changes every 2 days for totally 12 days, except fatty acid profile, which was determined at day 0 and 12 of storage. Sensory evaluation was conducted at day 0, 6 and 12 of storage.

5.3.6.1 Measurement of peroxide value (PV)

PV of sausage samples was determined according to the method of Richards and Hultin (2002) and Sakanaka *et al.* (2004). PV was expressed as μmol cumene hydroperoxide/kg sample.

5.3.6.2 Measurement of thiobarbituric acid-reactive substances (TBARS)

TBARS value was determined as described by Buege and Aust (1978). TBARS value was expressed as mg MDA/kg sample.

5.3.6.3 Measurement of fatty acid profiles

Fatty acid compositions were determined as fatty acid methyl esters (FAMEs) using a gas chromatography (GC-14A, Shimadzu Co., Kyoto, Japan) equipped with fused silica capillary column Carbowax-30 m (30 m, 0.25 mm ID,

Alltech Ltd., Deerfield, IL, USA) and flame ionization detector (FID) (Alltech Ltd., Deerfield, IL, USA). Helium was used as the carrier gas at a flow rate of 30 cm/s. The initial temperature of the column was set at 170 °C and increased to 225 °C with a rate of 1 °C/min and then held at 220 °C for an additional 20 min. The detector temperature was set at 270 °C, while the temperature at the injection port was maintained at 250 °C. Retention time of FAME standards was used to identify chromatographic peaks. Peak area was quantitated and expressed as% of total saponifiable lipids (AOAC, 2000).

5.3.6.4 Microbiological analysis

Fish sausage (25 g) was collected aseptically in a stomacher bag and 10 volumes of sterile saline solution (0.85%, w/v) were added. After homogenization in a Stomacher blender (Stomacher M400, Seward Ltd., Worthington, England) for 1 min, a series of 10-fold dilutions were made using normal saline solution (0.85%, w/v). Total viable count (TVC) and psychrophilic bacterial count (PBC) were determined by plate count agar (PCA) with the incubation at 37 °C for 2 days and 4 °C for 7 days, respectively (Maqsood *et al.*, 2012). Microbial counts were expressed as log CFU/g.

5.3.6.5 Texture profile analysis

Texture profile analysis (TPA) was performed using a TA-XT2 texture analyzer (Stable Micro Systems, Godalming, Surrey, UK) with load cell of 25 kg. Cylindrical aluminium probe (50 mm diameter) was used. The samples were cut into cylindrical-shape (30 mm height x 20 mm diameter) and placed on the instrument's base. The tests were run with two compression cycles. TPA textural parameters were measured at room temperature with following testing conditions: crosshead speed 5.0 mm/s, 50% compression of the original sample height, surface sensing force 99 g, threshold 30.0 g. Time interval between the first and second compressions was 10 s. Hardness, cohesiveness, springiness, chewiness and resilience were calculated from the force-time-curves generated for each sample.

5.3.6.6 Sensory evaluation

Sensory evaluation was performed by 30 untrained panellists (15 males and 15 females) with ages ranging from 20 to 35 years, who were familiar with the consumption of fish sausage. Before testing, the sausage samples were cooked and prepared as described by Maqsood and Benjakul (2012). Panellists were asked to evaluate for color, odor, appearance and overall likeness of sausage samples using a nine-point hedonic scale, in which a score of 1 = not like very much, 5 = neither like nor dislike and 9 = like extremely, respectively (Mailgaard *et al.*, 1999).

Samples were also evaluated for rancidity by 15 trained panellists (5 males and 10 females with ages ranging from 20-35 years) using a scale of 0 – 10, where 0 and 10 represent no rancidity and the strongest rancidity, respectively. Training was made as described by Thiansilakul *et al.* (2010).

5.3.7 Statistical analysis

All experiments were run in triplicate using three different lots of samples. Completely randomized design (CRD) was used throughout the study. Statistic analysis was performed using one-way analysis of variance (ANOVA). Mean comparison was carried out using Duncan's multiple range test (Steel and Torrie, 1980). Analysis was performed using the SPSS package (SPSS for windows, SPSS Inc., Chicago, IL, USA).

5.4 Results and discussion

5.4.1 Microstructure of emulsion sausage fortified with tuna roe lipids as affected by SRPH

Microstructures of PUFAs fortified emulsion sausage containing different amounts of SRPH are illustrated in Figure 15. Size of fat globules in the sausage was in a descending order: control (24-65 μm) > 0.5% SRPH (24-42μm) > 1% SRPH (15-25 μm) and 3% SRPH (10-20 μm). Increasing SRPH amounts more likely resulted in the formation of smaller fat globule with more uniform distribution. This was possibly due to emulsifying properties of SRPH, which formed interfacial

thin film around fat globules. After emulsification, SRPH along with myofibrillar proteins could migrate to the interface rapidly, thereby stabilizing emulsion formed. SRPH with small size peptides presumably exhibited superior emulsifying activity to larger proteins or peptides.

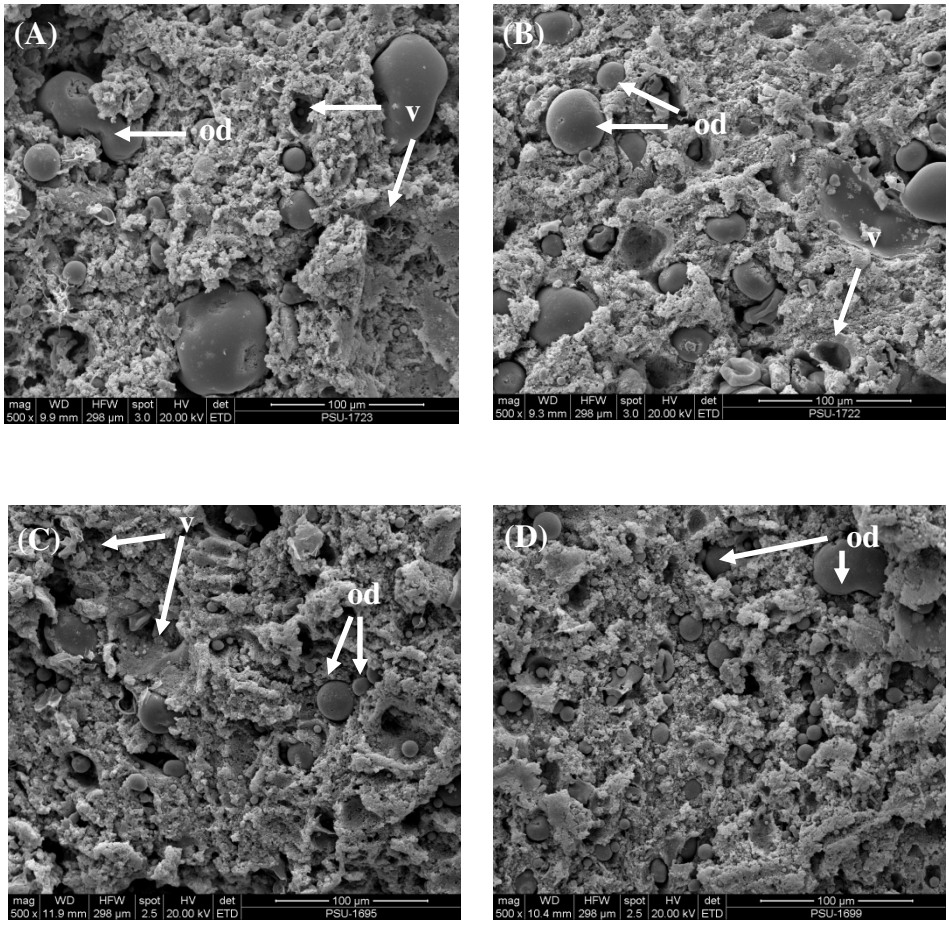


Figure 1 Scanning micrographs of broadhead catfish emulsion sausages fortified with skipjack tuna roe lipid containing SRPH at various levels (0-3%). Magnification: 500× (A); control (without SRPH), (B); sample added with 0.5% SRPH, (C); sample added with 1% SRPH and (D); sample added with 3% SRPH. v; Void and od: oil droplet.

Klompong *et al.* (2007) reported that the protein hydrolysate from yellow stripe trevally (*Selaroides leptolepis*) meat with the longer peptide chain exhibited the higher emulsifying activity. Youssef and Barbut (2010) suggested that high protein

matrix density, governed by higher degree of protein aggregation, adversely affected textural properties. Destabilization of emulsion is associated with the movement of fat and water towards the outside of the product (Youssef and Barbut, 2010). Thus, SRPH directly had the stabilizing impact on emulsion of sausage fortified with tuna roe lipids.

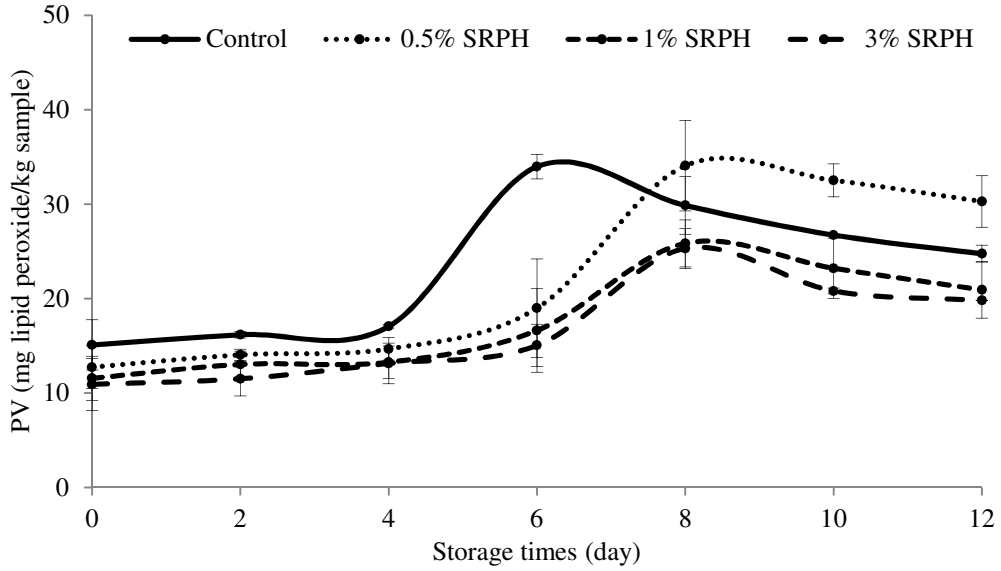
5.4.2 Changes in oxidative stability and properties of emulsion sausage fortified with tuna roe lipids

5.4.2.1 Lipid oxidation and fatty acid profiles

No marked changes in PV of emulsion sausage were observed in all samples during the first 4 days of storage ($p > 0.05$) (Figure 16A). The sharp increase in PV was noticeable in the control during 6 days of storage ($p < 0.05$). Thereafter, a decrease in PV was found ($p < 0.05$). The decreased PV observed with extended storage time was presumed to be due to the decomposition of hydroperoxides. Hydroperoxides underwent decomposition, yielding a wide variety of decomposition products, including aldehydes, ketone, acid, etc. (Chaijan *et al.*, 2006). Efficacy in preventing lipid oxidation of SRPH was dose-independent. However, PV of samples added with 1 and 3% SRPH was not different throughout the storage ($p > 0.05$). Thus, SRPH could act as antioxidant in emulsion sausage in conjunction with vitamin E present in sunflower oil. This result reconfirmed *in vitro* antioxidative activity of SRPH as reported by Intarasirisawat *et al.* (2012).

The increase in TBARS of all samples was noticeable throughout the refrigerated storage ($p < 0.05$) (Figure 16B). TBARS slightly increased within the first 4 days of storage. Thereafter, it increased abruptly up to 8 days for the control ($p < 0.05$). After 8 days, TBARS decreased until the end of storage. For samples added with SRPH, the continuous increase was found up to 10 days of storage, followed by the decrease at day 12 ($p < 0.05$). The decrease in TBARS during 8-10 days of storage was probably due to the loss in the volatile secondary oxidation products. Furthermore, those products might also react with amino acids to form the Schiff bases (Nordvi *et al.*, 2007b).

(A)



(B)

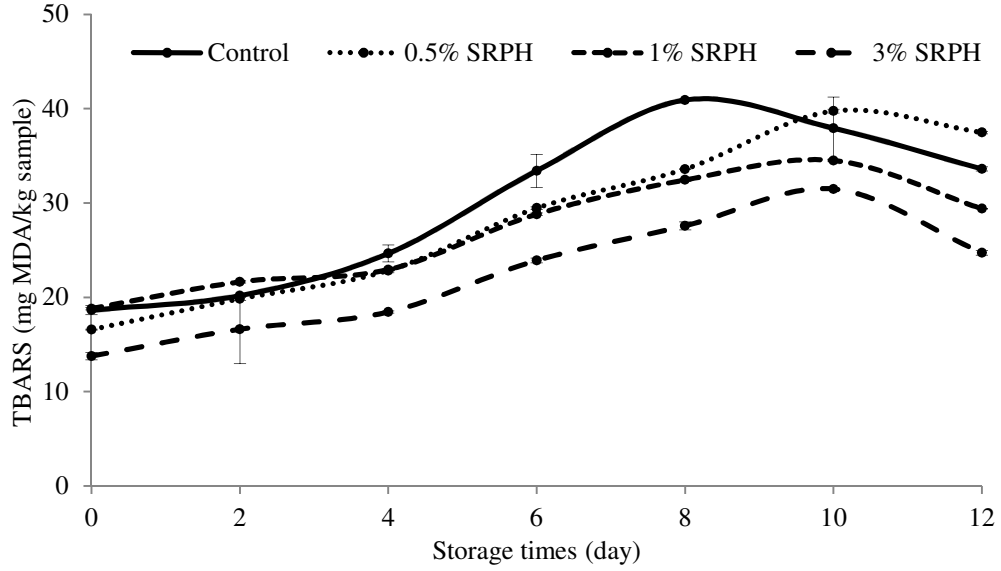


Figure 2 Effect of SRPH at various levels (0-3%) on PV (A) and TBARS (B) of broadhead catfish emulsion sausage during 12 days of refrigerated storage. Bar represents the standard deviation (n = 3).

At day 0, TBARs of all samples ranged from 13 to 22 mg MDA/kg of sample, indicating that the lipid oxidation occurred during processing and cooking of the sausages. This high TBARS value was associated with high proportion of PUFAs in

the emulsion sausage added with tuna roe lipids. Control sample showed the higher formation of TBARS during the storage of 12 days, when compared with other samples ($p < 0.05$), except the sample containing 0.5% SRPH, which had higher TBARS formation after 10 days of storage. The loss of volatile secondary products in the control sample at day 12 might be high, leading to the lower amount retained, compared with the amount found in the sample with 0.5% SRPH. The lowest TBARS value was observed in the sample added with 3% SRPH ($p < 0.05$). Apart from vitamin E, SRPH therefore more likely played a role in prevention of lipid oxidation in sausage incorporated with tuna roe lipids containing high PUFAs.

Fish sausage contained a wide range of fatty acids. Proportion of fatty acids in all sausage samples was in descending order: PUFA > MUFA > SFA. Linoleic acid (C18:2 n-6) was the dominant fatty acid, followed by oleic acid (C18:1 n-9) and palmitic acid (C16:0), respectively. n-3 fatty acids (DHA and EPA) was found at high content, accounting 8.93% of total saponifiable lipids. *Trans* fat including palmitelaidic acid methyl ester (C16:1 *n*9t) and elaidic acid (C18:1 *n*9t) were negligible. High content of linoleic acid was attributed to the addition of sunflower oil in the fish sausage. There were some changes in fatty acid composition of fish sausage enriched with PUFAs from tuna roe lipids in the presence of SRPH at different levels (0.5-3%) during refrigerated storage of 12 days (Table 13). Slight decrease in n-3 PUFA (DHA and EPA) of sample containing 1-3% SRPH was observed at day 12 of storage, while the higher decrease was noticeable in the control and the sample containing 0.5% SRPH. The decrease in EPA and DHA, especially in the control, might be due to their susceptibility to oxidation. The result was in accordance with the increase of TBARS and PV. As a result, the incorporation of SRPH into the n-3 PUFA fortified emulsion sausage prevented the loss of essential fatty acids, particularly n-3 PUFAs to some degree. During cold storage, the higher content of SFA was found in all samples. When PUFAs underwent oxidation, the ratio of saturated fatty acid slightly increased. The results suggested that SRPH was able to retard the oxidation of unsaturated fatty acids in emulsion sausage to some extent.

Table 1 Fatty acid profiles of broadhead catfish emulsion sausage added with SRPH at various levels before and after 12 days of storage

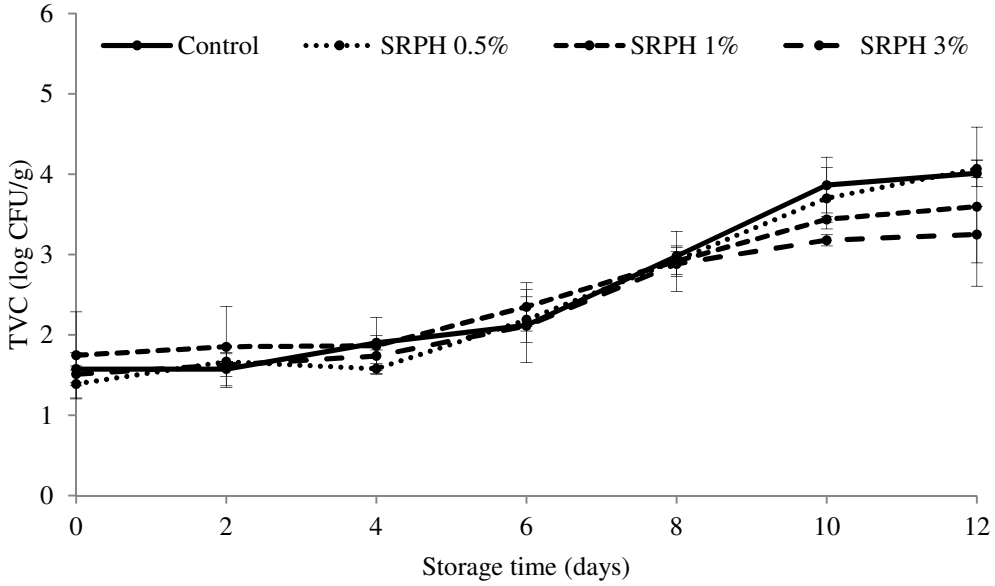
Fatty acids (% of total saponifiable lipid)	Day 0	Day 12			
		SRPH levels (g/ 100g)			
		Control	0.5	1	3
C12:0	0.06	0.08	0.06	0.07	0.06
C14:0	0.60	0.64	0.59	0.63	0.65
C14:1	0.18	0.02	0.02	0.02	0.02
C15:0	0.32	0.15	0.14	0.15	0.16
C16:0	14.00	15.94	15.24	15.80	15.86
C16:1 n9t	0.02	0.02	0.02	0.02	0.02
C16:1 n7	1.36	1.30	1.26	1.31	1.38
C17:0	0.22	0.24	0.23	0.25	0.26
C17:1	0.37	0.14	0.14	0.14	0.15
C18:0	5.52	6.04	5.94	5.99	5.94
C18:1 n9t	0.06	0.08	0.00	0.08	0.06
C18:1 n9	25.40	28.52	28.76	27.86	27.85
C18:1 n7	1.82	1.28	1.26	1.16	1.16
C18:2 n6	35.23	36.15	37.88	36.35	36.12
C18:3 n3	0.52	0.51	0.47	0.50	0.51
C18:3 n6	0.30	0.29	0.24	0.25	0.25
C18:4 n3	0.12	0.11	0.10	0.11	0.11
C20:0	0.22	0.19	0.20	0.19	0.18
C20:1 n7	0.03	0.02	0.02	0.02	0.02
C20:1 n9	0.49	0.48	0.45	0.45	0.44
C20:1 n11	0.53	0.06	0.06	0.06	0.06
C20:2 n6	0.03	0.25	0.22	0.23	0.23
C20:3 n6	0.32	0.35	0.30	0.33	0.32
C20:3 n3	0.04	0.04	0.03	0.04	0.04
C20:4 n6 AA	0.67	0.53	0.47	0.59	0.62
C20:4 n3	0.08	0.07	0.07	0.08	0.08
C20:5 n3 EPA	2.76	0.55	0.52	0.65	0.71
C21:0	0.01	0.02	0.01	0.00	0.00
C22:0	0.36	0.28	0.32	0.29	0.29
C22:1 n9	0.05	0.03	0.03	0.03	0.03
C22:1 n11	0.04	0.03	0.02	0.02	0.02
C22:4 n6	0.07	0.08	0.08	0.09	0.09
C22:5 n6	0.24	0.24	0.22	0.28	0.30
C22:6 n3 DHA	6.17	3.16	2.93	3.71	3.99
C23:0	0.05	0.05	0.05	0.05	0.05
C24:0	0.34	0.33	0.33	0.35	0.36
C24:1	0.05	0.04	0.05	0.05	0.05
Unidentified peak	1.34	1.49	1.11	1.65	1.37
SFA	21.70	23.95	23.11	23.77	23.80
MUFA	30.41	32.04	32.09	31.22	31.27
PUFA	46.55	42.34	43.53	43.19	43.38
SFA/PUFA ratio	0.47	0.57	0.53	0.55	0.55
n-3	9.69	4.44	4.12	5.07	5.45
n-6	36.87	37.89	39.41	38.11	37.93
n-9	26.00	29.11	29.24	28.41	28.37
n-3/n-6 ratio	0.26	0.12	0.10	0.13	0.14

Values are mean ± SD (n = 2).

5.4.2.2 Changes in microbial load

TVC and PBC of the sausage samples were monitored during storage (Figure 17). TVC was constant during the first 4 days of storage, followed by the gradual increase up to day 10. For PBC, it was not detected within the first 2 days. After 2 days of storage, the rapid growth of PBC was noticeable and PBC was higher than TVC, indicating that psychrophilic bacteria was dominant and more likely became the major cause in the spoilage of emulsion sausages. TVC over than 5 log CFU/g indicates the deterioration and unacceptance of meat products (Stannard, 1997). Thus, shelf-life of broadhead catfish emulsion sausages was approximately 10-12 days based on the limit for TVC and PBC. Similar result was also reported for the cooked catfish sausage added with catfish bone hydrolysate (CBH) at different concentrations (0.5-2.0%) during storage at room temperature (Ren *et al.*, 2011). The addition of CBH with concentration of 1.5-2.0 g/100g could retard microbial growth when compared with other treatments ($p < 0.05$) (Ren *et al.*, 2011). Traditionally, ingredients including nitrite and spices (garlic, onion, curmin, pepper, eg.) are used to preserve sausage quality owing to their antibacterial activity (Panpipat and Yongsawatdigul, 2008; Rahman *et al.*, 2007). Essential oils and phenolic compounds from plant extracts could delay the proliferation of aerobic plate counts and could extend the shelf-life of products. Incorporation of tannic acid and kiam wood extract was reported to extend the shelf-life of striped catfish sausages (Maqsood *et al.*, 2012). For PBC, the lower count was observed in sample added with 3% SRPH during 4-6 days and 10-12 days, compared with others. However, there was no difference in PBC between samples added with 1% and 3% SRPH at the end of storage ($p > 0.05$). It has been reported that some peptides exhibited antimicrobial activity (Ren *et al.*, 2011; Song *et al.*, 2012). However, SRPH generally could not effectively extend the shelf-life of broadhead catfish emulsion sausage. Therefore the preservative or antimicrobial substances may be needed.

(A)



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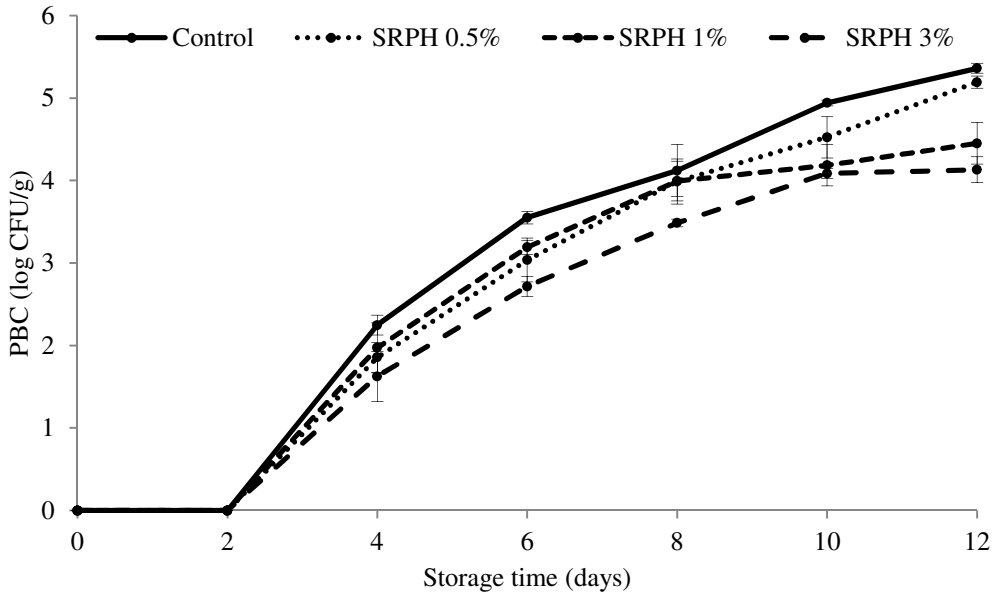


Figure 3 Total viable count (A) and psychrophilic bacterial count (B) of broadhead catfish emulsion sausages during 12 days of refrigerated storage. Bars represents the standard deviation (n = 3).

5.4.2.3 Changes in textural properties

Changes in textural properties of the cooked fish emulsion sausages during the storage are shown in Table 14. The addition of SRPH at all levels increased hardness, chewiness and resilience of the samples ($p < 0.05$). However, it showed no impact on springiness of samples ($p > 0.05$). The addition of SRPH could increase cohesiveness at day 0 of storage, but there was no difference in cohesiveness among all samples during the storage ($p > 0.05$).

Table 2 Texture profiles of broadhead catfish emulsion sausages added with SRPH at different levels during refrigerated storage

Storage time (days)	SRPH levels (%)	Texture profile analysis (TPA)				
		Hardness (N)	Cohesiveness	Springiness	Chewiness (N)	Resilience
0	Control	30.24 ± 1.15^{Bc}	0.34 ± 0.03^{Bb}	0.93 ± 0.01^{Aa}	9.65 ± 0.99^{Bc}	0.16 ± 0.01^{Bb}
	0.5	46.91 ± 0.82^{Ab}	0.52 ± 0.01^{Ab}	0.92 ± 0.02^{ABa}	22.23 ± 0.60^{Ac}	0.34 ± 0.03^{Aa}
	1	47.99 ± 0.73^{Aa}	0.53 ± 0.01^{Aa}	0.90 ± 0.00^{Ca}	23.58 ± 0.44^{Aa}	0.33 ± 0.01^{Ab}
	3	48.14 ± 3.64^{Aa}	0.53 ± 0.02^{Aa}	0.91 ± 0.01^{BCa}	23.51 ± 2.40^{Aa}	0.34 ± 0.02^{Aa}
6	Control	41.77 ± 2.33^{Bb}	0.52 ± 0.01^{Aa}	0.92 ± 0.01^{ABa}	20.37 ± 1.70^{Bb}	0.32 ± 0.01^{Ca}
	0.5	47.37 ± 0.93^{Ab}	0.53 ± 0.01^{Aa}	0.92 ± 0.01^{Aa}	23.35 ± 0.39^{Ab}	0.34 ± 0.01^{BCa}
	1	48.91 ± 1.70^{Aab}	0.53 ± 0.01^{Aa}	0.90 ± 0.01^{Ba}	24.19 ± 1.51^{Aa}	0.33 ± 0.00^{Bb}
	3	48.96 ± 0.83^{Aa}	0.53 ± 0.01^{Aa}	0.91 ± 0.01^{ABa}	24.11 ± 0.31^{Aa}	0.35 ± 0.01^{Aa}
12	Control	48.31 ± 1.16^{Ba}	0.52 ± 0.00^{Aa}	0.90 ± 0.01^{Ab}	22.41 ± 0.90^{Ba}	0.32 ± 0.01^{Ba}
	0.5	49.93 ± 0.40^{Aa}	0.53 ± 0.01^{Aa}	0.90 ± 0.01^{Aa}	24.62 ± 0.58^{Aa}	0.35 ± 0.00^{Aa}
	1	49.89 ± 1.22^{Aa}	0.52 ± 0.01^{Aa}	0.90 ± 0.02^{Aa}	24.24 ± 0.85^{Aa}	0.35 ± 0.00^{Aa}
	3	49.94 ± 1.05^{Aa}	0.52 ± 0.00^{Aa}	0.91 ± 0.00^{Aa}	24.22 ± 0.16^{Aa}	0.35 ± 0.00^{Aa}

Values are mean ± SD (n = 3).

Different uppercase superscripts within the same column in the same storage time indicate the significant differences ($p < 0.05$).

Different lowercase superscripts within the same column in the same SRPH level indicate the significant differences ($p < 0.05$).

Among all samples containing SRPH at various levels (0.5 – 3%), there was no difference in force required to compress sample to attain a given deformation

(hardness), capability in breaking down the internal structure (cohesiveness) and the required energy to chew the sample to the point required for swallowing it (chewiness). In general, the level of SRPH did not affect the textural properties of sausage samples. It implied that SRPH in the range used in this study might exhibit emulsifying activity, in which emulsion could be stabilized in the matrix. As storage time increased to 12 days, the control sample had a marked increase in hardness and chewiness from 30.24 to 48.31 N and from 9.65 to 22.41 N, respectively ($p < 0.05$). The increase in resilience was also found in the control with increasing storage time ($p < 0.05$). This might be plausibly due to the greater oxidative damage of proteins in the control sample, which led to the formation of protein cross-links (Eymard *et al.*, 2009). As a consequence, the increased hardness and chewiness and the decreased springiness of fish emulsion sausage were obtained.

Lipid and protein oxidations are relatively associated with the deterioration occurring in meat product (Mercier *et al.*, 2004). SRPH was able to retard the lipid oxidation of fish emulsion sausage during the storage to some degree. The result suggested that textural properties of fish emulsion sausage could be stabilized over the time of storage with the addition of SRPH at 0.5%.

5.4.2.4 Changes in sensory properties

Color, odor, appearance and overall likeness of the broadhead catfish emulsion sausage added with different amounts of SRPH (0-3%) during storage were scored by 30-untrained panellists as shown in Table 15. There were no differences in all attributes among all samples ($p > 0.05$) at day 0 of storage. The result indicated that SRPH did not affect sensory properties of fish emulsion sausage. The incorporation of other natural antioxidants such as kiam wood extract (0.04 and 0.08%) and tannic acid (0.02 and 0.04%) (Maqsood *et al.*, 2012), lutein, ellagic acid and sesamol (0.20, 0.30 and 0.25%) (Hayes *et al.*, 2011) into emulsion sausage also had no impact on consumer likeness. The addition of 20 and 40 g/kg dietary fish oil to chicken frankfurters had no negative effect on flavor or acceptability of chicken frankfurters (Jeun-Horng *et al.*, 2002).

Table 3 Likeness score of broadhead catfish emulsion sausages added with SRPH at various levels during refrigerated storage

Storage time (day)	SRPH levels (%)	Likeness score			
		Color	Odor	Appearance	Overall
0	Control	7.29 ± 0.78^{Aa}	7.09 ± 0.70^{Aa}	7.18 ± 1.08^{Aa}	7.00 ± 1.00^{Aa}
	0.5	7.14 ± 0.69^{Aa}	7.00 ± 0.89^{Aa}	7.18 ± 0.98^{Aa}	7.00 ± 0.77^{Aa}
	1	7.57 ± 0.98^{Aa}	7.00 ± 0.77^{Aa}	7.18 ± 1.08^{Aa}	7.00 ± 1.00^{Aa}
	3	7.43 ± 0.53^{Aa}	6.91 ± 0.94^{Aa}	7.18 ± 0.60^{Aa}	6.91 ± 1.14^{Aa}
6	Control	7.00 ± 0.63^{Aab}	6.67 ± 1.03^{Aa}	6.82 ± 0.87^{Aa}	5.82 ± 1.25^{Bb}
	0.5	7.36 ± 0.81^{Aa}	6.86 ± 1.21^{Aa}	7.09 ± 0.54^{Aa}	6.36 ± 0.92^{ABbc}
	1	7.27 ± 0.65^{Aa}	7.00 ± 1.19^{Aa}	7.18 ± 0.87^{Aa}	6.82 ± 0.92^{ABa}
	3	7.27 ± 0.90^{Aa}	6.82 ± 0.87^{Aa}	7.18 ± 0.87^{Aa}	6.73 ± 0.65^{Aa}
12	Control	6.57 ± 1.31^{Ab}	6.00 ± 1.26^{Ab}	6.73 ± 1.19^{Aa}	4.91 ± 1.22^{Bc}
	0.5	6.71 ± 1.38^{Aa}	6.17 ± 1.33^{Ab}	6.82 ± 0.75^{Aa}	6.09 ± 1.04^{Ac}
	1	7.00 ± 1.04^{Aa}	6.50 ± 0.55^{Aa}	7.18 ± 0.87^{Aa}	6.36 ± 0.50^{Ab}
	3	7.00 ± 1.19^{Aa}	6.41 ± 0.44^{Aa}	7.09 ± 0.94^{Aa}	6.18 ± 0.25^{Ab}

Values are mean ± SD (n = 30).

Different uppercase superscripts within the same column in the same storage time indicate the significant differences ($p < 0.05$).

Different lowercase superscripts within the same column in the same SRPH level indicate the significant differences ($p < 0.05$).

Score 1 = dislike extremely; 5 = neither like nor dislike; 9 = like extremely.

Generally, likeness score for all attributes decreases with increasing storage time. Nevertheless, the degree of decrease varied with samples. At day 12 of storage, the control showed the lowest color likeness score, compared with others ($p < 0.05$). The sample added with 1 and 3% SRPH had the higher odor and overall likeness, compared with others ($p < 0.05$). This was in accordance with the lowered oxidation taken place in the samples added with SRPH at higher levels.

Changes in rancidity of fish emulsion sausage added without and with SRPH at various levels were monitored during storage (Table 16). During the storage, higher rancidity was found in the control at all storage times tested ($p < 0.05$). At day

12, the control sample and that containing 0.5% SRPH showed the higher rancidity than others ($p < 0.05$). Similar rancidity was found between samples added with 1 and 3% SRPH ($p > 0.05$). The result was in agreement with the likeness score (Table 3). In food systems, the release of odor or flavor compounds is also implicated with other constituents such as carbohydrates, hydrocolloids and proteins. Rancidity in processed meat products causes the changes in odor, flavor, taste, color, texture and appearance (Hayes *et al.*, 2011).

Table 4 Rancidity of broadhead catfish emulsion sausage added with SRPH at various levels during refrigerated storage

SRPH levels (%)	Storage time (days)		
	0	6	12
Control	1.34 ± 0.28^{Ac}	3.55 ± 0.35^{Ab}	5.53 ± 0.41^{Aa}
0.5	1.25 ± 0.27^{Ac}	2.84 ± 0.22^{Bb}	5.30 ± 0.24^{Aa}
1	1.01 ± 0.06^{Ac}	2.53 ± 0.50^{Bb}	3.68 ± 0.15^{Ba}
3	1.28 ± 0.03^{Ac}	2.36 ± 0.10^{Bb}	3.65 ± 0.28^{Ba}

Values are mean ± SD (n = 15).

Different uppercase superscripts within the same column indicate the significant differences ($p < 0.05$).

Different lowercase superscripts in the same row indicate the significant differences ($p < 0.05$).

Additionally, lipid oxidation is engaged with unhealthiness due to free-radical production in the body, which can cause health problems. Therefore, delaying lipid oxidation can have a significant contribution towards prevention of nutritional loss and lowering the risk of health problem (Hayes *et al.*, 2011). These results indicated that SRPH could be incorporated into fish emulsion sausages without having a detrimental effect on organoleptic quality of sausage and be able to maintain sensory properties by retarding the development of rancid odor.

5.5 Conclusion

Broadhead catfish emulsion sausages, enriched with n-3fatty acid from skipjack tuna roe, were susceptible to oxidation during 12 days of refrigerated storage. SRPH was used as the natural antioxidant to retard lipid oxidation in emulsion sausage during the refrigerated storage. Thus, PUFA was more retained in the sausage added with SRPH. Addition of SRPH could improve textural properties but had no detrimental effect on the organoleptic properties. Therefore, SRPH having 5% DH, especially at a level of 3%, can be applied to improve the hardness and extend the shelf-life of fish sausage.

CHAPTER 6

Influence of high pressure homogenization on stability of emulsions containing skipjack roe protein hydrolysate

6.1 Abstract

The effect of high pressure homogenization at various levels (2,000, 3,000 and 4,000 psi) on stability of emulsions containing skipjack roe protein hydrolysate (SRPH) and sodium caseinate was comparatively studied during 14 days of storage. Higher homogenization pressure resulted in the higher decrease in particle sizes as evidenced by the decreased volume frequency distribution (d_{43}) ($p < 0.05$). The protein concentration at interface was increased with increasing homogenization pressures ($p < 0.05$). Dominant interfacial proteins of SRPH stabilized emulsions had the molecular weight in the range of 7.0-16.4 kDa. During the extended storage, particle size, creaming index, flocculation factor (F_f) and coalescence index (C_i) of SRPH stabilized emulsions sharply increased, especially in emulsions prepared at 3,000 and 4,000 psi ($p < 0.05$). Nevertheless, emulsions containing SRPH showed the lower stability, compared with those stabilized by sodium caseinate ($p < 0.05$). Therefore, the stability of emulsion stabilized by SRPH could be improved by homogenization at an appropriate pressure (2,000 psi).

6.2 Introduction

High pressure homogenization mechanically reduces the size of particles, producing emulsion with homogeneity and high stability (Lee *et al.*, 2009). High-pressure homogenization can disrupt the flocculated clusters, thereby dispersing agglomerates uniformly. Combination of intense shear, cavitation and turbulent flow conditions increases the surface activity of emulsifying molecules (Bouaouina *et al.*, 2006; Jafari *et al.*, 2008c; Marco-Molés *et al.*, 2012). Two main emulsifiers, amphiphilic macromolecules (mainly proteins) and low molecular weight emulsifiers (lecithins, monoglycerides, tweens, spans, etc. (Yun and Hong, 2007), have been widely used. During the emulsification process, proteins unfold, migrate, orientate

and adsorb at oil droplet surface. Those proteins can form multilayers surrounding the dispersed particles, thereby strengthening steric hindrance, and reducing the rate of coalescence (Khan *et al.*, 2014). Additionally, the creaming velocity is proportional to the square of the droplet diameter and density difference (Robins, 2000). Thus, the decreased average size of the oil droplet attributed by high pressure homogenization can reduce the creaming velocity (Stokes' law), and therefore increases the stability of emulsion. However, the excessive pressure and time used for homogenization was reported to produce emulsion with the larger average droplet size, called "overprocessing" (Hebishy *et al.*, 2013; Jafari *et al.*, 2007).

Recently, a new food-grade natural emulsifier has gained a considerable attention. Skipjack tuna roe, a by-product of tuna canning industry, contained phosvitin and lipovitellin, which can serve as an alternative emulsifier with nutritive value (Intarasirisawat *et al.*, 2011). However, these proteins are naturally formed as insoluble granule of lipovitellin-phosvitin complex. To recover those proteins and increase their solubility, enzymatic hydrolysis has been implemented. Skipjack roe protein hydrolysate with degree of hydrolysis (DH) of 5% using Alcalase was found to have the emulsifying property (Intarasirisawat *et al.*, 2012). The implementation of high pressure to reduce the size of droplets and to favor the localization of peptides at interface can be a promising approach to improve the stability of emulsion containing roe protein hydrolysate. To the best of our knowledge, no information regarding the effect of high pressure homogenization on stability of emulsion stabilized by roe protein hydrolysate has been reported. Thus, the aim of this study was to elucidate the impact of homogenization at varying pressure levels on stability of emulsion containing skipjack roe protein hydrolysate.

6.3 Materials and methods

6.3.1 Chemicals

Sodium azide (NaN_3) and saccharose were purchased from Fluka Chemical (Buchs, Switerland). Acridine orange, Nile blue A and sodium dodecyl sulfate (SDS) were bought from Sigma Chemical Co. (St. Louis, MO, USA).

Coomassie brilliant blue R-250 was obtained from Merck (Darmstadt, Germany). All reagents were of analytical grade except soybean oil, which was of commercial grade (Thanakorn Vegetable Oil Products Co. Ltd., Samut prakan, Thailand).

6.3.2 Preparation of protein hydrolysate

Frozen roes of skipjack (*Katsuwonus pelamis*) obtained from Songkhla Canning Company (Ltd), Songkhla, Thailand, were thawed and defatted as described by Intarasirisawat *et al.* (2011). Protein hydrolysate with 5% DH was prepared using Alcalase as per the method of Intarasirisawat *et al.* (2012). The obtained skipjack roe protein hydrolysate (SRPH) was lyophylized using a freeze-dryer (CoolSafe 55, ScanLaf A/S, Lynge, Denmark), placed in polyethylene bag and kept at -20 °C until use.

6.3.3 Effect of high pressure homogenization on emulsion characteristic and stability

Emulsion was prepared according to the method of Castellani *et al.* (2006) with a slight modification. SRPH (5 g) was dispersed into 100 mL of distilled water and then adjusted pH to 7.0 using 1 M HCl. Ten milliliters of soybean oil were added with 100 mL of SRPH solution (oil volume fraction: 0.1). The mixture was homogenized at a speed of 10,000 rpm for 2 min using a homogenizer (Model T25 basic, IKA LABORTECHNIK, Selangor, Malaysia). The coarse emulsions were then passed through a Microfluidics homogenizer (Model HC-5000, Microfluidizer, Newton, MA, USA) at different pressures (2,000, 3,000 and 4,000 psi) for fifteen passes. NaN_3 (0.02%, w/v) was added to the emulsions as an antimicrobial agent. The control emulsion was prepared in the same manner using 5% (w/v) sodium caseinate. All emulsion samples were then stored at room temperature (28-30 °C) for 14 days. The samples were taken at day 1, 7 and 14 for analyses, except for creaming index and microstructure analyses. Creaming index was monitored at day 1, 3, 5, 7, 9, 11 and 14 and the microstructure was examined at day 1 and 14.

6.3.4 Analyses

6.3.4.1 Particle size distribution

Particle size distribution of emulsions was determined using a liquid particle size analyser (LPSA) (Model LS 230, Beckman Coulter®, Fullerton, CA, USA) as per the method of Castellani *et al.* (2006) with a slight modification. Prior to analysis, an aliquot of emulsion (5 mL) was diluted with 1% (w/v) sodium dodecyl sulfate (SDS) solution (20 mL) in order to dissociate flocculated droplets. The surface-weighted mean particle diameter (d_{32}) and the volume-weighted mean particle diameter (d_{43}) of the emulsion droplets were measured.

6.3.4.2 Flocculation and coalescence

To determine flocculation factor and coalescence index, the emulsions were diluted with distilled water in the presence and absence of 1% (w/v) SDS. The flocculation factor (F_f) and coalescence index (Ci) were calculated using the following equations:

$$F_f = \frac{d_{43\text{-}SDS}}{d_{43+\ SDS}}$$

$$C_i = \frac{(d_{43+SDS\ ,\ t} - d_{43+SDS,in}) \text{ x}100}{d_{43+SDS,in}}$$

where d_{43+SDS} and $d_{43\text{-}SDS}$ are the volume-weighted mean particle diameter of the emulsion droplets in the presence and absence of 1% SDS, respectively; $d_{43+SDS,in}$ and $d_{43+SDS,t}$ are the volume-weighted mean particle diameter of the emulsion droplets in the presence of 1% SDS at the designated storage time.

6.3.4.3 ζ-potential

The electrical charge (ζ-potential) of oil droplets in the emulsions was determined using a ZetaPlus zeta potential analyser (Brookhaven Instruments Corporation, Holtsville, NY, USA) at room temperature. The ζ-potential was

determined by measuring the direction and velocity of droplet movement in the applied electric field. The ζ-potential of each individual sample was calculated.

6.3.4.4 Creaming

Creaming was measured according to the method of Keowmaneechai and McClements (2002) with a slight modification. The emulsions (13 mL) were poured into test tubes and stored at room temperature. The volume of the separated aqueous phase at the bottom of the tube was recorded. Creaming was calculated as follows:

Creaming% = (Height of clear droplet-free phase/ Total height of the emulsion) x 100

Creaming was monitored via the kinetic formation of a clear droplet-free phase at the bottom of the sample. The percentage of creaming was plotted against storage times (0, 1, 3, 5, 7, 9, 11 and 14 days).

6.3.4.5 Confocal laser scanning microscopy

Behaviors of emulsion were examined with a confocal laser scanning microscope (CLSM) (Olympus, FV300, Tokyo, Japan). The emulsion (100 µL) was suspended with 20 µL of 0.01% (w/v) Nile blue A and 20 µL of 0.01% (w/v) acridine orange in order to label lipid and protein, respectively. Five microliters of prepared samples were smeared on the microscopy slide. The CLSM was operated in the fluorescence mode at the excitation wavelength of 533 nm and the emission wavelength of 630 nm using a Helium Neon Red laser (HeNe-R) for lipid analysis and at the excitation wavelength of 488 nm and the emission wavelength of 540 nm using a Helium Neon Green laser for protein analysis (Guérin-Dubiard *et al.*, 2002). Magnification of 200x was used.

6.3.4.6 Interfacial protein concentration

Interfacial protein concentration of emulsions was determined according to the method of Patton and Huston (1986). Emulsion was diluted with 50% (w/v) saccharose in the same buffer of the emulsion aqueous phase at 1:1 ratio (v/v).

An aliquot of mixture (7 mL) was then carefully deposited at the bottom of a centrifuging tube containing 13.5 mL of a 5% (w/v) saccharose solution in the corresponding buffer. These tubes were centrifuged at 3,000g for 2 h at 10 °C and then immediately frozen at -20 °C for 24 h. The frozen tube (-20 °C) were cut to recover the two phases. Upper phase including creamed oil droplets at the top and an intermediate separating phase, and lower phase or the aqueous phase located at the bottom were obtained. The protein content was determined in all fractions. Proteins in the upper phase and lower phase were adsorbed and unadsorbed proteins, respectively. The protein content of the turbid middle phase was included as the adsorbed protein. Interfacial protein concentration (Γ, mg/m^2) was calculated as follows:

$$\Gamma = \frac{\text{Adsorbed protein concentration (mg/mL of oil)}}{\text{Specific surface area } S_s \text{ (m}^2\text{/mL of oil)}}$$

The specific surface area of oil droplets (S_s in m^2/mL of oil) was calculated from the surface weighted mean particle diameter (d_{32} in mm) according to Walstra (1983) as shown below:

$$S_s = 6/d_{32}$$

6.3.4.7 Protein patterns

Total protein and interfacial protein were subjected to sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) using 15% separating gel and 4% stacking gel according to the method of Laemmli (1970). Samples were diluted in sample buffer (0.25 M Tris–HCl pH 6.8, 0.04% bromophenol blue and 30% glycerol, and 6% SDS solution) to obtain designated protein concentration. Proteins (18 μg) determined by the Lowry's method (Lowry *et al.*, 1951) was 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 separation, the proteins were stained with 0.02% (w/v) Coomassie Brilliant Blue R-

250 in 50% (v/v) methanol and 7.5% (v/v) acetic acid for 3 h and destained with 50% (v/v) methanol and 7.5% (v/v) acetic acid for 15 min, followed by 5% (v/v) methanol and 7.5% (v/v) acetic acid for 3 h. Low molecular weight markers were used for estimation of MW of protein bands.

6.3.5 Statistical analysis

All experiments were run in triplicate. All analyses were conducted in five replications, except for interfacial protein concentration and creaming index, which were performed 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 and Torrie, 1980). Analysis was performed using the SPSS package (SPSS for windows, SPSS Inc., Chicago, IL, USA).

6.4 Results and discussion

6.4.1 Particle size distribution

Particle size of emulsions containing SRPH and sodium caseinate prepared with different pressures (2,000-4,000 psi) expressed as d_{32} and d_{43} was monitored during 14 days of storage at room temperature (Table 17). With increasing homogenization pressure, the emulsions stabilized by SRPH or sodium caseinate had the lower d_{32} ($p > 0.05$) and d_{43} ($p < 0.05$). Generally, emulsions stabilized by SRPH possessed the larger size of droplets, compared with those containing sodium caseinate. At day 1 of storage, the SRPH emulsions had d_{32} of 0.32-0.36 µm and d_{43} of 0.57-1.14 µm. For emulsions with sodium caseinate, d_{32} of 0.16-0.20 µm and d_{43} of 0.28-0.36 µm were obtained. d_{32} and d_{43} are more sensitive to the presence of small and large particles, respectively (Surh *et al.*, 2006). The d_{32} is inversely proportional to specific surface area. The smaller d_{32} contributes to the higher specific surface area, which offers the increase in protein loads for adsorbing at interface of emulsions (Hebishy *et al.*, 2013). The d_{43} can be used as the index of coalescence and flocculation. The increase in d_{43} reflects the association of individual droplets into larger droplet (Hebishy *et al.*, 2013). Particle size distribution is an important

parameter involving in physical properties (color, viscosity and texture) and shelf-life of food emulsion (McClements, 2005).

Table 1 Particle size of droplets in emulsions stabilized by SRPH and sodium caseinate prepared using different pressures during storage.

Sample	Pressure used (psi)	Storage time (days)	d_{32} (μm)	d_{43} (μm)
SRPH	2,000	1	0.36±0.07 ^{Ac}	1.14±0.03 ^{Ac}
		7	1.75±0.06 ^{Ab}	2.60±0.02 ^{Ab}
		14	2.14±0.03 ^{Aa}	3.00±0.01 ^{Aa}
	3,000	1	0.32±0.09 ^{Ab}	0.67±0.12 ^{Bc}
		7	1.18±0.08 ^{Ca}	2.01±0.01 ^{Bb}
		14	1.36±0.09 ^{Ca}	2.39±0.02 ^{Ba}
	4,000	1	0.34±0.07 ^{Ac}	0.57±0.11 ^{Bc}
		7	1.37±0.05 ^{Bb}	1.96±0.00 ^{Cb}
		14	1.77±0.02 ^{Ba}	2.24±0.01 ^{Ca}
Sodium caseinate	2,000	1	0.20±0.01 ^{Ab}	0.36±0.02 ^{Aa}
		7	0.31±0.04 ^{Aa}	0.37±0.01 ^{Aa}
		14	0.28±0.02 ^{Aa}	0.38±0.01 ^{Aa}
	3,000	1	0.20±0.02 ^{Aa}	0.32±0.05 ^{ABa}
		7	0.23±0.05 ^{Ba}	0.28±0.03 ^{Ba}
		14	0.23±0.03 ^{Ba}	0.27±0.02 ^{Ca}
	4,000	1	0.16±0.02 ^{Bb}	0.28±0.05 ^{Ba}
		7	0.22±0.05 ^{Ba}	0.33±0.04 ^{Aa}
		14	0.25±0.02 ^{Ba}	0.31±0.01 ^{Ba}

Values are mean ± SD (n = 5)

Different lowercase superscripts in the same column within the same pressure and sample indicate significant difference ($p < 0.05$).

Different uppercase superscripts in the same column within the same storage time and sample indicate significant difference ($p < 0.05$).

During 14 days of storage, d_{32} and d_{43} of emulsion stabilized by SRPH were increased ($p < 0.05$), suggesting the coalescence of oil droplets. On the other hand, sodium caseinate could maintain the oil droplet size in emulsion during the extended storage. Differences in emulsifying properties between SRPH and sodium

caseinate were plausibly due to the different constituents, conformation, hydrophobicity and chain length of peptides or proteins (Hebishy *et al.*, 2013). The employment of high pressure technique for homogenization caused the modification of protein conformation, particularly globular protein (Wang *et al.*, 2008). Those proteins or peptides with more exposed hydrophobic domains likely adsorbed at interface of droplet more effectively. Adsorption of the modified macromolecule of sodium caseinate surrounding interfacial oil droplet provided steric hindrance against coalescence (Sánchez and Patino, 2005). However, Floury *et al.* (2004) suggested that an excessive pressure used may cause the detrimental effect on emulsifying properties of globular protein. Excessive unfolding of globular protein induced by high pressure could affect the interfacial properties of globular protein, thus enhancing coalescence. Puppo *et al* (2005) reported that emulsion containing soybean protein isolate (10 mg/mL) homogenized with high pressures (14 – 87,022 psi) had particle size (d_{43}) with the range of 0.91-1.57 μm, which was most likely higher than droplet sizes observed in this study. This was possibly due to the difference in protein concentration, protein constituents, amphiphilic character, flexibility of emulsifying molecules and interfacial film rheology (Romero *et al.*, 2009). Thus, particle size of droplets in emulsions was affected by emulsifiers and pressure used for homogenization.

6.4.2 Flocculation and coalescence

Stability of emulsions was monitored in term of flocculation factor (F_f) and coalescence index (C_i) during 14 days of storage at room temperature as shown in Table 18. For emulsions containing SRPH, the increase in pressure for homogenization resulted in the increase in F_f of emulsion after one day of storage ($p < 0.05$). For coalescence index, the increase in pressure yielded emulsion with increased coalescence index. Homogenization pressure presumably affected the interfacial properties of the emulsifiers used. The application of higher pressure might change the conformation of the emulsifiers at interface (Molina *et al.*, 2001).

Table 2 Flocculation, coalescence and ζ-potential of emulsions prepared by using different pressures during storage

Sample	Pressure used (psi)	Storage time (days)	Flocculation factor (F_f)	Coalescence index(C_i)	Z-potential (mV)
SRPH	2,000	1	1.71±0.04 ^{Cb}	-	-41.63±1.36 ^{Aa}
		7	2.30±0.02 ^{Ca}	128.82±1.69^{C}	-38.92±1.09 ^{Ab}
		14	2.33±0.02 ^{Ca}	164.06±0.64^{C}	-37.22±0.91 ^{Ac}
	3,000	1	2.76±0.34 ^{Ba}	-	-40.33±1.00 ^{Aa}
		7	2.74±0.01 ^{Ba}	201.05±1.19^{B}	-39.51±1.00 ^{Aa}
		14	2.92±0.03 ^{Ba}	257.44±2.37^{B}	-37.55±0.95 ^{Ab}
	4,000	1	3.36±0.08 ^{Ab}	-	-40.37±0.79 ^{Aa}
		7	3.30±0.07 ^{Ab}	242.42±0.62^{A}	-36.05±1.39 ^{Aa}
		14	3.89±0.07 ^{Aa}	291.59±1.07^{A}	-35.98±2.72 ^{Ab}
Sodium caseinate	2,000	1	1.10±0.04 ^{Bb}	-	-52.70±2.04 ^{Bb}
		7	1.60±0.04 ^{Aa}	2.46±0.23^{C}	-51.09±2.86 ^{Aab}
		14	1.63±0.08 ^{Aa}	3.35±0.18^{B}	-49.03±2.45 ^{Ab}
	3,000	1	1.21±0.04 ^{Aa}	-	-54.02±2.82 ^{Bab}
		7	1.18±0.05 ^{Ba}	3.08±0.29^{B}	-51.40±0.72 ^{Ab}
		14	1.19±0.04 ^{Ba}	17.48±1.76^{A}	-51.20±1.33 ^{Ab}
	4,000	1	1.12±0.02 ^{Bab}	-	-56.58±2.62 ^{Aa}
		7	1.07±0.06 ^{Cb}	9.82±0.40^{A}	-51.10±1.05 ^{Ab}
		14	1.14±0.02 ^{Ba}	18.09±0.76^{A}	-51.13±1.36 ^{Ab}

Values are mean ± SD (n = 5)

Different lowercase superscripts in the same column within the same pressure and sample indicate significant difference ($p < 0.05$).

Different uppercase superscripts in the same column within the same storage time and sample indicate significant difference ($p < 0.05$).

This could lead to lower ability to decrease the interfacial tension. Therefore, the increase in F_f and C_i could be noticed when higher homogenization pressure was employed. With increasing storage time, the flocculation and coalescence increased with all samples, especially in emulsions stabilized by SRPH ($p < 0.05$). The lower F_f and C_i of emulsions containing sodium caseinate indicated higher stability of

emulsions. The ability in adsorbing at the oil-water interface, forming matrix around oil droplet and reducing interfacial tension between particles determined emulsifying property of proteins (Hebishy *et al.*, 2013). When storage time increased, emulsions with SRPH prepared using 2,000 psi and 4,000 psi had the increase in F_f ($p < 0.05$). However, no difference in F_f was found in emulsion with 3,000 psi with increasing storage time. Similar trend was noticeable with emulsions containing sodium caseinate. The results suggested that pressures used for homogenization affected the stability of emulsion. The coalescence index indicated that emulsion with highest stability can be achieved by using homogenization pressure of 2,000 psi of both SRPH and sodium caseinate.

6.4.3 Creaming index

Creaming indexes of emulsions containing sodium caseinate and SRPH homogenized with different pressures as a function storage time are shown in Figure 18. Within the first 7 days, emulsions containing SRPH, prepared using pressure of 2,000 and 3,000 psi rendered the higher creaming index than that of 4,000 psi. Thereafter, no difference in creaming was found in all samples after day 9 of storage. The increase in creaming was related with increased flocculation and coalescence (Table 18) indicating rapid creaming happen in flocculated and coarser emulsion (Sánchez and Patino, 2005). For emulsions containing sodium caseinate, creaming increased within the first 3 days of storage. Subsequently, no further change in creaming index was observed until the end of storage. This was in agreement with the smaller mean droplet diameter (Table 17) and lower F_f and C_i (Table 18). Creaming is an undesirable phenomenon, in which buoyant emulsion droplets form at the top of emulsion (Sánchez and Patino, 2005). Since the separation of cream phase indicates the instability, an appropriated pressure for emulsification was required. The lower creaming rate of emulsion prepared by sodium caseinate could be explained by Stokes' law, where the decrease of average size of oil droplet contributes to the reduction of creaming velocity (Khan *et al.*, 2014).

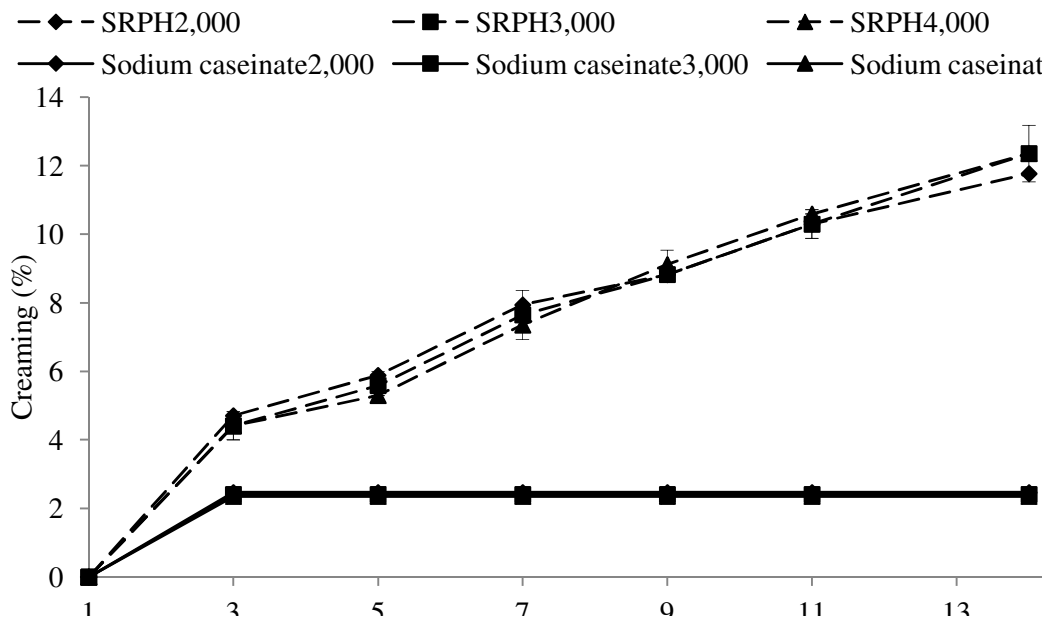


Figure 1 Change of creaming during 14 days of storage. Bars represent the standard deviation (n=3). Numbers denote the level of pressure for homogenization (psi).

6.4.4 ζ-potential

ζ-potential of emulsions containing SRPH and sodium caseinate as affected by homogenization pressures is shown in Table 18. At the first day of storage, emulsion samples had ζ-potential values lower than -40 mV. Emulsion stabilized by sodium caseinate had ζ- potential values lower than -50 mV. Negatively charged residues on oil droplet mostly contributed to repulsion between droplets, thereby lowering coalescence. ζ-potential is the potential difference between the dispersion medium and the stationary layer of fluid attached to the dispersed droplet (Wang *et al.*, 2011c). Emulsions exhibiting absolute ζ-potential higher than +30 mV or lower than −30 mV tend to be electrostatically stable, while emulsions within the range of (-30) - 30 mV tend to coagulate or flocculate (Wang *et al.*, 2011c). It was noted that emulsion stabilized with sodium caseinate having higher ζ-potential showed higher stability. With increasing storage time, ζ-potential of all samples became decreased, especially emulsions containing SRPH. During the extended storage, the layers of protein surrounding droplets might undergo aggregation via

ionic interaction as indicated by the change in ζ-potential. The insufficient electrostatic repulsion might lead to the development of flocculation and coalescence, particularly with extended storage times. Particle size of the resulting emulsions and the stability of emulsion were governed by ζ-potential surrounding droplets, which was more likely associated with the charge of proteins at interface.

6.4.5 CLSM micrograph

Particle distribution of emulsions stabilized by SRPH or sodium caseinate, prepared with different homogenization pressures, was visualized by CLSM (Figure 19). At the first day of storage, CLSM images depicted the particulate clusters or clumps of oil droplets (red color) in SRPH stabilized emulsions. It was noted that the smallest droplet size was found in emulsion prepared with 3,000 psi, compared with those observed in emulsions with pressures of 2,000 and 4,000 psi. Level of pressure applied for emulsification of sunflower oil emulsion affected distribution and size of oil droplets (Marco-Molés *et al.*, 2012). However, no flocs were noticeable in emulsions stabilized by sodium caseinate, regardless of pressures applied. At day 14 of storage, it was found that flocculation and coalescence took place in SRPH stabilized emulsions, particularly samples with 3,000 and 4,000 psi. No flocculation and coalescence were observed in emulsion stabilized by sodium caseinate. Disruption of emulsion indicated by CLSM images was in accordance with the increases in d_{32}, d_{43} (Table 17), F_f and *Ci* (Table18), when storage time increased. This reflected the higher stability of emulsion containing sodium caseinate as an emulsifier.

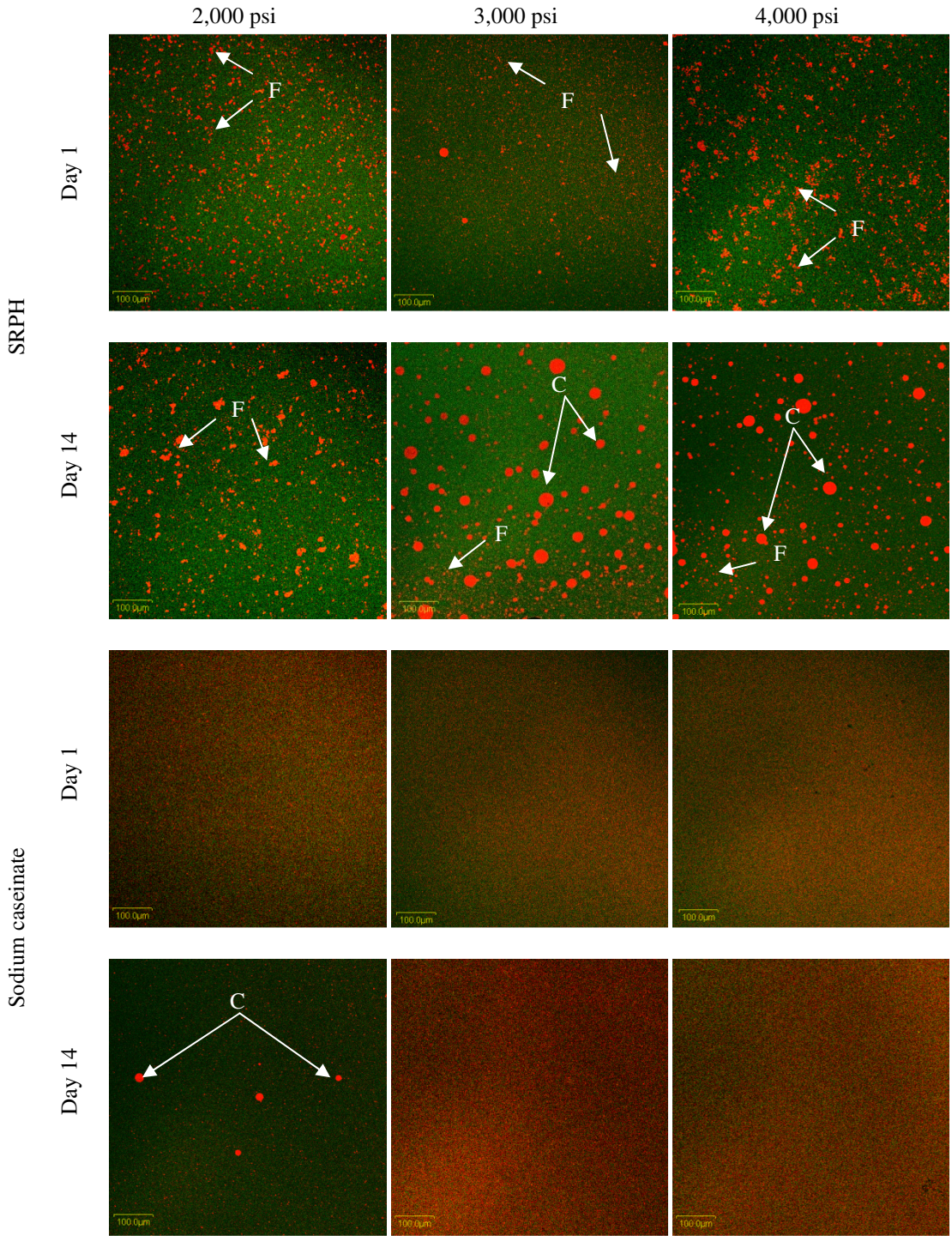


Figure 2 Droplet distribution of emulsions stabilized by SRPH and sodium caseinate prepared using various pressure levels. Magnification; 200x. Red and green represent lipid and protein, respectively. F; flocculated droplet, C; coalesced droplet.

6.4.6 Interfacial protein concentration

Interfacial protein concentration (Γ, mg/m^2) of emulsion stabilized by SRPH and sodium caseinate using different homogenization pressures is presented in Table 19. Interfacial protein or adsorbed protein at interfacial area reflects the adsorption characteristic of SRPH and sodium caseinate at oil droplet interface. Generally, the employment of high pressure homogenization can dissociate oil droplets into small particles with the increased surface area. Thus, higher amount of protein load per surface area is required for coating the newly created interface (Hebishy *et al.*, 2013). Concentration of protein at interface was influenced by surface area and size (d_{32}) of the oil droplets. High Γ-values of emulsions with the decreased oil surface area were coincidental with the increased d_{32}. The decreased Γ-values could be observed for emulsions having small d_{32} (increased oil surface area). The rearrangement of protein or peptide layer might be thinner and probably approaching a monolayer (Damodaran, 2005; Srinivasan *et al.*, 1996). The increase in Γ-values of the emulsions stabilized by SRPH was found with increasing homogenization pressure and storage time. The increases in Γ-values during storage were related with increasing coalescence (Table 18). Slightly decreased Γ-values were observed for emulsions stabilized by sodium caseinate. The difference in interfacial protein level might be governed by different molecular structure, size and rearrangement of adsorbed protein molecules at the interface (Taherian *et al.*, 2011). The higher Γ-values of emulsions containing SRPH, compared with those containing sodium caseinate, might be due to the fact that shorter peptides of SRPH could not reorient at the interface in the way that stabilized emulsions effectively. Consequently, coalescence could occur and the lower surface of oil droplet was obtained as indicated by increased d_{32} (Table 17). The protein concentration at the oil-water interface was the crucial factor in the stability of the emulsions because proteins can lower the interfacial tension at the oil-water interface of the droplets. High pressure homogenization may induce protein association or aggregation between protein in the aqueous phase and the protein that previously formed monomolecular layer at interface (Khan *et al.*, 2014). For SRPH stabilized emulsions, high pressure above

2,000 psi may induce the peptide aggregation, as indicated by lowered ζ-potential value. Thus, the decrease in emulsion stability was observed.

Table 3 Interfacial protein concentration

Sample	Pressure used (psi)	Storage time (day)	Interfacial protein concentration (mg/m^2)
SRPH	2,000	1	1.38 ± 0.07^{Ca}
		7	1.83 ± 0.03^{Ba}
		14	1.77 ± 0.31^{Ba}
	3,000	1	1.59 ± 0.04^{Bb}
		7	2.43 ± 0.01^{Aa}
		14	2.54 ± 0.13^{Aa}
	4,000	1	1.75 ± 0.10^{Ab}
		7	1.83 ± 0.01^{Bb}
		14	2.21 ± 0.18^{ABa}
Sodium caseinate	2,000	1	0.31 ± 0.01^{Ac}
		7	0.46 ± 0.00^{Aa}
		14	0.35 ± 0.01^{Ab}
	3,000	1	0.27 ± 0.00^{Ba}
		7	0.26 ± 0.01^{Bb}
		14	0.20 ± 0.00^{Bc}
	4,000	1	0.16 ± 0.01^{Cc}
		7	0.23 ± 0.00^{Cb}
		14	0.26 ± 0.00^{Ca}

Values are mean ± SD (n = 3)

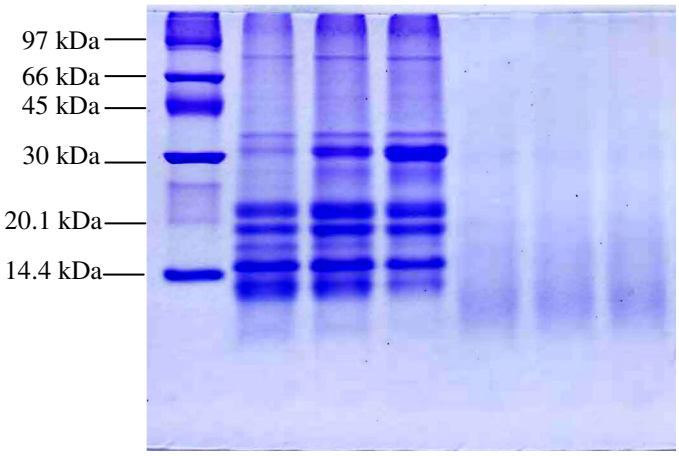
Different lowercase superscripts in the same column within the same pressure and sample indicate significant difference ($p < 0.05$).

Different uppercase superscripts in the same column within the same storage time and sample indicate significant difference ($p < 0.05$).

6.4.7 SDS-PAGE

Patterns of proteins and interfacial proteins under non-reducing condition are shown in Figure 20A and Figure 20B, respectively.

(A)



(B)

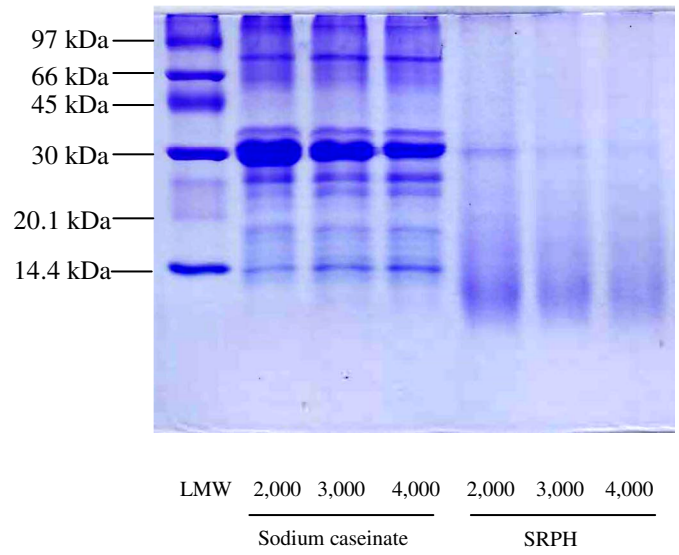


Figure 3 Electrophoretic patterns of total protein (A) and interfacial protein (B) of sodium caseinate and skipjack roe protein hydrolysate (SRPH) containing emulsions prepared using different pressures. Samples were loaded onto 15% running gel and 4% stacking gel in the absence of β-mercaptoethanol. LMW: Low molecular weight marker

In general, sodium caseinate contains four major proteins including α_{s1}-casein (~23 kDa), α_{s2}-casein (~25 kDa), *β*-casein (~29 kDa) and κ-casein (~19 kDa), whereas, SRPH had major peptides with MW of 5.5 and 57 kDa (Intarasirisawat et. al., 2012). From Figure 20A, the absence of protein with MW of 57 kDA in SRPH with 5% DH

was observed. Protein with MW of 57 kDa might be stabilized via ionic interiaction or hydrophobic effect, leading to dissociation of this protein in the presence of SDS. Protein and interfacial protein in emulsion stabilized by SRPH and sodium caseinate were slightly different. Higher band intensity of protein with MW of 30 kDa increased with increasing homogenization pressure (Figure 20A). High pressure induced the rupture of non-covalent interactions between protein molecules, followed by the reformation of intra - and intermolecular bonds within or between protein molecules (Bouaouina *et al.*, 2006) via disulfide linkage (He *et al.*, 2013). Coincidentally, proteins with MW less than 14 kDa decreased. With higher pressure, shearing became pronounced. As a result, those proteins might undergo cross-link via disulfide bond.

For interfacial protein composition, proteins with MW of 30 kDa showed the slight decrease in intensity when the higher pressure was applied for homogenization (Figure 20B). Cross-linked protein plausibly migrated to the interface more slowly, compared with the smaller protein. Therefore, proteins especially at interface were influenced by pressure applied. This affected the localization of protein and stiffness of protein films at interface.

6.5 Conclusion

Stability of emulsion correlated with the homogenization pressure. Higher homogenization pressure reduced droplet size but decreased amount of adsorbed proteins. Emulsification at 2,000 psi could provide the highest stability of SRPH containing emulsion during 14 days of storage. However, SRPH having 5% DH had poorer emulsifying properties than sodium caseinate. Therefore, the improvement of emulsifying properties of SRPH is still required.

CHAPTER 7

Stability of emulsion containing skipjack roe protein hydrolysate modified by oxidized tannic acid

7.1 Abstract

Stability of menhaden oil-in-water emulsion incorporated with skipjack roe protein hydrolysate (SRPH) at different levels (5 and 10%, w/v) was determined when oxidized tannic acid (OTA) at a level of 1% (w/v) was added before and after emulsification. During 14 days of storage, the addition of OTA yielded SRPH-emulsions with larger particle sizes (d_{43} and d_{32}), but less coalescence index (C_i) and flocculation factor (F_f), compared to those without OTA, regardless of OTA incorporation stage. Among all samples, emulsion stabilized by 10% SRPH showed the lower coalescence and flocculation, when 1% OTA was added after emulsification ($p < 0.05$). The stability of SRPH-emulsion added with OTA after emulsification as a function of OTA concentrations (0-2%, w/w) was assessed. When OTA concentrations increased, d_{43} and d_{32}, C_i and F_f decreased but ζ-potential value increased. Smaller droplets with less coalescence were obtained with increasing OTA concentrations. OTA slightly induced cross linking of peptides, particularly those located at the interface. SRPH-emulsion containing OTA inhibited the formation of TBARS in a dose-dependent manner ($p < 0.05$). Therefore, SRPH along with OTA incorporation after emulsification could increase stability of emulsion and enhanced the oxidative stability.

7.2 Introduction

Marine oil is a rich source of polyunsaturated fatty acids (PUFAs), especially ω-3 and ω-6 fatty acids, which have been claimed for their health benefits (Ramakrishnan *et al.*, 2013a; Sahena *et al.*, 2009). Nevertheless, the uses of marine oil in a processed food are limited, owing to its susceptibility to oxidation (Jacobsen *et al.*, 2008). For emulsion, the appropriate coating surrounding oil droplets in the system is another alternative means to lower oxidation (Dalgleish, 2006). Proteins are

extensively used as emulsifiers in food products because they can decrease interfacial tension between oil and aqueous phase and form a continuous viscoelastic membrane-like film around oil droplets (Aewsiri *et al.*, 2009). Additionally, protein film surrounding oil droplets can play a role in prevention of lipid oxidation of oil droplets (Aewsiri *et al.*, 2009). Some amino acids in proteins are able to scavenge free radical and chelate prooxidative metals (Djordjevic *et al.*, 2008).

Phenolic compounds from various plants have been reported to prevent lipid oxidation in fish oil-in-water emulsion, corresponding to their antioxidative properties such as radical scavenging, iron chelating, and reducing activities (Salminen *et al.*, 2010; Sekhon-Loodu *et al.*, 2013). Nevertheless, the phenolic compounds in oxidized form may exert pro-oxidant in initiating free radical chain reaction (Michalak, 2006). Additionally, the oxidized phenolics can partially loss their reducing power or antioxidative activity (Aewsiri *et al.*, 2010). However, they act as protein cross-linker (Aewsiri *et al.*, 2013; Balange and Benjakul, 2009). The oxidized phenolic compounds are able to react with nucleophilic groups of several amino acids such as tryptophan, cysteine, methionine, histidine, tyrosine and proline, thereby inducing the cross-linking via those reactive groups (Kroll *et al.*, 2003). Aewsiri *et al.* (2009) reported that gelatin modified with oxidized tannic acid (OTA) via covalent interaction rendered the emulsion with high stability and could inhibit lipid oxidation of menhaden oil-in-water emulsion effectively throughout the storage of 12 days.

Proteins from egg yolk including lipovitellin and phosvitin have been demonstrated as the potential emulsifiers (Daimer and Kulozik, 2009). Hydrophobicity of lipovitellin (lipoprotein) is involved in interfacial property, whereas repulsive force caused by phosphate moieties of phosvitin (phosphoprotein) favors the emulsion stability (Samaraweera *et al.*, 2011). Nevertheless, lipovitellin-phosvitin complex is less soluble and hydrolysis was implemented to increase solubility. Alcalase digestion was applied to skipjack roe in order to recover these proteins and augmented the solubility (Intarasirisawat *et al.*, 2012). Skipjack roe protein hydrolysate having 5% degree of hydrolysate (DH) showed high emulsifying properties accompanied with antioxidative activity. Although high pressure homogenization at 2,000 psi could improve the stability of emulsion containing SRPH

with 5% DH, the separated phases were also noticeable during extended storage (Intarasirisawat *et al.*, 2014). To enhance stability of emulsion containing SRPH, the modification of protein films using phenolic compounds, particularly oxidized form, might increase emulsion stability via introducing the stronger film surrounding oil droplets. The incorporation stage of phenolic compounds into emulsion could also affect in emulsifying property of protein or hydrolysate. Furthermore, phenolic compounds with retained reducing capacity could function as antioxidant in the emulsion system. Those could contribute to enhanced stability. Therefore, the aim of this study was to investigate the characteristics and oxidative stability of menhaden oil-in-water emulsion stabilized by SRPH as influenced by OTA incorporation before and after emulsification.

7.3 Materials and methods

7.3.1 Chemicals/oil

Sodium azide (NaN_3) was purchased from Fluka Chemical (Buchs, Switerland). Acridine orange, Nile blue A, tannic acid, menhaden oil and sodium dodecyl sulfate (SDS) were procured from Sigma Chemical Co. (St. Louis, MO, USA). Coomassie brilliant blue R-250 was obtained from Merck (Darmstadt, Germany). All reagents were of analytical grade.

7.3.2 Preparation of oxidized tannic acid and skipjack roe protein hydrolysate

Oxidized tannic acid (OTA) was prepared according to the method of Aewsiri *et al.* (2009). Tannic acid (2%, w/v) was dissolved in distilled water, followed by a pH adjustment to 9 using 1 M NaOH. Solution was then bubbled with high purity oxygen (99.5%) (Thai Industrial Gases PCL, Songkhla, Thailand) at 40 °C for 1 h for conversion of tannic acid to OTA with 70.15±2% degree of conversion.

Skipjack roe protein hydrolysate (SRPH) was prepared as per the method of Intarasirisawat *et al.* (2012) to obtain a degree of hydrolysis (DH) of 5%. Briefly, the defatted roe was suspended in distilled water to obtain protein

concentration of 20 mg/mL. The mixture was homogenized and pre-incubated at 50 °C for 20 min. The hydrolysis reaction was initiated by addition of Alcalase. The reaction was conducted at pH 8.0 and 50 °C for 1h. The enzymatic reaction was terminated by heating at 85 °C for 15 min. DH of the resulting hydrolysate was measured as per the method of Benjakul and Morrissey (1997). The obtained skipjack roe protein hydrolysate (SRPH) with pH 6.98 was lyophylized using a freeze-dryer (CoolSafe 55, ScanLaf A/S, Lynge, Denmark), placed in polyethylene bag and kept at -20 °C until use. SRPH was dissolved in distilled water and then adjusted to pH 9 using 1 M NaOH.

7.3.3 Preparation of emulsions

SRPH solutions with the concentrations of 5 and 10% (w/v) were emulsified with menhaden oil (oil volume fraction of 0.1) at a speed of 10,000 rpm for 2 min using a homogenizer (Model T25 basic, IKA LABORTECHNIK, Selangor, Malaysia). The coarse emulsions were then passed through a Microfluidics homogenizer (Model HC-5000, Microfluidizer, Newton, MA, USA) at the pressure of 2,000 psi with fifteen passes. NaN_3 (0.02%, w/w) was added to the emulsions as an antimicrobial agent. Oil-in-water emulsions referred to as 'SRPH-emulsion' were stored at room temperature (26-28 °C). The samples were taken at day 1, 7 and 14 for analyses except for confocal laser scanning microscopy (CLSM), in which the samples were taken at day 1 and 14 of storage.

7.3.4 Effect of OTA incorporation on emulsion stability

OTA was added into emulsion containing SRPH (5 and 10%, w/v) to obtain the concentration of 1% (based on SRPH content). The addition of OTA was performed before and after emulsification. The resulting emulsions were termed 'pre-emulsified' and 'post-emulsified', respectively. To prepare pre-emulsified sample, SRPH solutions (pH 9.0) were added with OTA solution (pH 9.0) and the mixtures were continuously stirred using a magnetic stirrer (RO 10 power IKAMAG®, IKA LABORTECHNIK, Selangor, Malaysia) at 200 rpm at room temperature for 12 h.

Thereafter, the mixtures were added with menhaden oil. Coarse and fine emulsions were prepared sequentially as described above.

For post-emulsified sample, coarse emulsion containing SRPH without OTA was subjected to high-pressure homogenization with thirteen passes. Thereafter, OTA solution was added and the mixtures were homogenized for another two passes. The control for both processes was prepared in the same manner except OTA was excluded. The positive controls including emulsions containing Na-caseinate or Tween 20 at levels of 5 and 10% (w/v) were also prepared. The emulsion samples were kept at room temperature and taken for analyses. Emulsion yielding the low C_i and F_f was selected for further study.

7.3.5 Effect of OTA concentration on emulsion stability

Post-emulsified samples containing OTA at different concentrations (0, 0.1, 0.5, 1 and 2%, based on SRPH content) were prepared. The positive controls including emulsions containing Na-caseinate or Tween 20 at a level of 10% (w/v) in the absence of OTA were also prepared. Emulsions were stored at room temperature and taken for analyses.

7.3.6 Analyses

7.3.6.1 Particle size distribution

Particle size distribution of emulsions was determined using a liquid particle size analyser (LPSA) (Model LS 230, Beckman Coulter®, Fullerton, CA, USA) as per the method of Castellani *et al.* (2006). Prior to analysis, an aliquot of emulsion (5 mL) was diluted with 1% (w/v) sodium dodecyl sulfate (SDS) solution (20 mL) in order to dissociate flocculated droplets. The surface-weighted mean particle diameter (d_{32}) and the volume-weighted mean particle diameter (d_{43}) of the emulsion droplets were measured.

7.3.6.2 Flocculation and coalescence

Emulsions were diluted with distilled water in the presence and absence of 1% (w/v) SDS. The flocculation factor (F_f) and coalescence index (Ci) were calculated using the following equations:

$$F_f = \frac{d_{43\text{-}SDS}}{d_{43+SDS}}$$

$$Ci = \frac{(d_{43+SDS\,,\,t} - d_{43+SDS,in})}{d_{43+SDS,in}} \text{ x}100$$

where d_{43+SDS} and $d_{43\text{-}SDS}$ are the volume-weighted mean particle diameter of the emulsion droplets in the presence and absence of 1% SDS, respectively; $d_{43+SDS,in}$ and $d_{43+SDS,t}$ are the volume-weighted mean particle diameter of the emulsion droplets in the presence of 1% SDS at time 0 and the designated storage time.

7.3.6.3 ζ-potential

The electrical charge (ζ-potential) of oil droplets in the emulsions was determined using a ZetaPlus zeta potential analyser (Brookhaven Instruments Corporation, Holtsville, NY, USA) at room temperature. The ζ-potential of each individual sample was measured.

7.3.6.4 Confocal laser scanning microscopy

Microstructures of emulsion were examined with a confocal laser scanning microscope (CLSM) (Olympus, FV300, Tokyo, Japan). The emulsion (100 μL) was suspended with 20 μL of Nile blue A and 20 μL of acridine orange in order to label lipid and protein, respectively. Five microliters of prepared samples were smeared on the microscopy slide. The CLSM was operated in the fluorescence mode at the excitation wavelength of 533 nm and the emission wavelength of 630 nm using a Helium Neon Red laser (HeNe-R) for lipid analysis and at the excitation wavelength of 488 nm and the emission wavelength of 540 nm using a Helium Neon Green laser for protein analysis (Guérin-Dubiard *et al.*, 2002). Magnification of 600x was used.

7.3.6.5 Total protein and adsorbed protein

Total protein and adsorbed protein of emulsions were prepared according to the method of Patton and Huston (1986). Briefly, emulsion was suspended in 50% (w/v) saccharose at 1:1 ratio (v/v) (total protein). An aliquot of mixture (7 mL) was then carefully deposited at the bottom of a centrifuging tube containing 13.5 mL of a 5% (w/v) saccharose solution in the corresponding buffer. These tubes were centrifuged at 3,000g for 2 h at 10 °C and then immediately frozen at -20 °C for 24 h. The frozen tube (-20 °C) were cut to recover the upper phases, which was referred to as 'an adsorbed protein fraction'. The amount of adsorbed protein was measured using Lowry method (Lowry *et al.*, 1951) and calculated according to the method of Perrier-Cornet *et al.* (2005) as following equation;

$$\text{Adsorbed protein (\%)} = \frac{\text{adsorbed protein (g) x 100}}{\text{total protein (g)}}$$

7.3.6.6 Protein patterns

Total protein and adsorbed protein fractions obtained from previously described were subjected to sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) using 15% separating gel and 4% stacking gel according to the method of Laemmli (1970). Total protein and adsorbed protein of samples were diluted in sample buffers (0.25 M Tris–HCl pH 6.8, 0.04% bromophenol blue and 30% glycerol, and 6% SDS solution with and without 10% βME) to obtain designated protein concentration. Proteins (18 μg) determined by the Lowry's method (Lowry *et al.*, 1951)was 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 separation, the proteins were stained with 0.02% (w/v) Coomassie Brilliant Blue R-250 in 50% (v/v) methanol and 7.5% (v/v) acetic acid for 3 h and destained with 50% (v/v) methanol and 7.5% (v/v) acetic acid for 15 min, followed by 5% (v/v) methanol and 7.5% (v/v) acetic acid for 3h. Low range molecular weight markers were used for estimation of MW of protein bands.

7.3.6.7 Oxidative stability

The emulsions were stored at 30 °C in the dark and taken for thiobarbituric acid reactive substances (TBARS) analysis at day 1, 3, 5, 7, 9, 12 and 14. TBARS were determined as described by Buege and Aust (1978). Two milliliters of emulsion sample were mixed with 10 mL of thiobarbituric acid solution (0.375% thiobarbituric acid, 15% trichloroacetic acid and 0.25 M HCl). The mixture was heated in boiling water for 10 min, followed by cooling in running tap water. The mixture was centrifuged at 3,600 xg for 20 min at room temperature. The absorbance of supernatant was read at 532 nm. The standard curve was prepared using malondialdehyde (MDA) (2-10 ppm). TBARS were expressed as mg MDA/L emulsion.

7.3.7 Statistical analysis

All experiments were run in triplicate using three lots of samples. All analyses were conducted in five replications except for TBARS analysis, which was performed in triplicate. Data was subjected to one-way analysis of variance (ANOVA). Mean comparison was carried out using Duncan's multiple range test (Steel and Torrie, 1980). For pair comparison, T-test was used (Steel and Torrie, 1980). Analysis was performed using the SPSS package (SPSS for windows, SPSS Inc., Chicago, IL, USA).

7.4 Results and discussion

7.4.1 Effect of OTA with pre- and post-emulsification on physical stability of emulsion containing SRPH

Emulsions containing SRPH at various concentrations (5 and 10%, w/v) containing 1% OTA were prepared with pre- or post-emulsifications. Changes in particle size distribution, coalescence and flocculation of the obtained emulsions were monitored during storage of 14 days.

7.4.1.1 Particle size distribution

Particle size distribution of emulsion stabilized by SRPH at different concentrations in the presence and absence of OTA as influenced by pre- and post emulsification is shown in Table 20. The d_{32} is inversely related to specific surface area. The smaller d_{32} means the higher specific surface area, offering the increase in protein loads at interface of emulsions (Hebishy *et al.*, 2013). The d_{43} can be used as the index of coalescence and flocculation (Hebishy *et al.*, 2013). The increase in d_{43} reflects the assembly of individual droplets into larger flocs (Hebishy *et al.*, 2013; McClements, 2005). Particle size distribution is an important parameter involving in physical properties (color, viscosity and texture) and shelf-life of food emulsion (Huck-Iriart *et al.*, 2013). Without OTA incorporation, emulsion containing 10% SRPH showed the larger oil droplets (d_{43} and d_{32}) than that with 5% SRPH throughout the storage ($p < 0.05$). The larger particle size of the former might be attributed to self-assembly of SRPH and bridging effects (Gudipati *et al.*, 2010). This lowered the ability of SRPH to migrate and localize at the interface. Thus, the excessive amount of SRPH showed the adverse effect of emulsifying property. When OTA was incorporated into emulsion with pre- and post-emulsification, it was noted that SRPH concentration and emulsification stage had the high impact on emulsion. With emulsion containing 5% SRPH, OTA addition with pre-emulsification gave higher d_{32} and d_{43} than that without OTA incorporation. When OTA was added, the cross-linking of peptides or proteins might take place. This led to the increase in size, thereby lowering the migration of those peptides to interface. For emulsion containing 10% SRPH, the incorporation of OTA with either pre- or post-emulsification rendered the smaller particle size (d_{32} and d_{43}) than that without OTA incorporation ($p < 0.05$). The incorporation of OTA not only induced the cross-linking but also reduced the self-assembly of SRPH, particularly at level of 10%. This led to adequate amount of peptides anchored at the interface and formed strong film surrounding oil droplet. As a result, the lower d_{32} and d_{43} could be observed. When comparing between pre- and post-emulsification, the latter yielded the lower d_{32} and d_{43} for emulsion containing 5% SRPH. When peptides were localized at interface and formed film surrounding oil droplet during emulsification, the subsequent incorporation of OTA (post-

emulsification) might help in cross-linking the pre-occupied film surrounding oil droplet. This could lower coalescence of emulsion as indicated by lower d_{43}. When emulsion contains 10% SRPH, the addition of OTA with post-emulsification yielded emulsion with higher d_{43}, compared to that with pre-emulsification. *Ortho*-quinone of OTA more likely interacted with SRPH. The larger aggregates more likely lost emulsifying property as indicated by the increased d_{43}. Nevertheless, when the adsorbed protein was determined, it was found that the emulsion containing 10% SRPH showed the higher level of adsorbed proteins, 50.88 and 58.53% for pre-emulsification and post-emulsification, respectively, when compared with the emulsion containing 5% SRPH, either for pre-emulsification and post-emulsification (45.09 and 45.24%, respectively). The higher thickness also contributed to the larger d_{43}. The thicker layer of film surrounding the droplet could form the bridges between different droplets, as indicated by the increase in flocculation. The quinone forms a dimer in a side reaction, or reacts with amino or sulfhydryl side chains of polypeptides to form covalent C–N or C–S bonds with the phenolic ring. The addition of OTA with post-emulsification might therefore induce some cross-linking of SRPH, particularly those occupied at interface during pre-emulsification. This could help in physical stabilization of the film surrounding oil droplets to some degree. When comparing emulsion containing SRPH with those having Tween 20 and Na-caseinate as emulsifiers, it was found that both Tween 20 and Na-caseinate yielded the emulsion with smaller oil droplets as evidenced by lower d_{32} and d_{43}. Tween 20 has been used as the common non-ionic surfactant in emulsion (Burch *et al.*, 2010), while Na-caseinate has been known as the protein with high emulsifying property (Ye, 2011).

During the storage, the increases in particle size, both d_{32} and d_{43}, were noticeable in all emulsion samples. This indicated the instability of emulsion, in which the collapse of emulsion by coalescence mechanism might occur. At day 14 of storage, d_{32} and d_{43} sharply increased for emulsions containing 5% SRPH, whereas slight increases in d_{32} and d_{43} were observed for SRPH-emulsion containing OTA with post-emulsification.

Table 1 Particle size of droplets in emulsions stabilized by SRPH as affected by pre- and post-emulsification during storage of 14 days

Samples	Processes	Storage time (days)	d_{32} (μm)	d_{43} (μm)
5% SRPH	No OTA	1	0.25±0.00 ^{Bb}	1.18±0.00 ^{Cb}
		7	0.54±0.02 ^{Cb}	2.85±0.18 ^{Cb}
		14	1.98±0.12 ^{Bb}	3.31±0.04 ^{Cb}
	1% OTA/ Pre-emulsification	1	0.72±0.00 ^{Aa}	2.08±0.08 ^{Ba}
		7	1.25±0.03 ^{Aa}	4.23±0.04 ^{Aa}
		14	2.34±0.06 ^{Aa}	5.18±0.02 ^{Aa}
	1% OTA/ Post-emulsification	1	0.58±0.02 ^{Ab}	2.63±0.23 ^{Aa}
		7	0.69±0.02 ^{Bb}	3.41±0.00 ^{Ba}
		14	1.92 ±0.13^{Bb}	4.66±0.03 ^{Ba}
5% Tween 20	No OTA	1	0.21±0.00 ^{Cb}	0.37±0.02 ^{Cb}
		7	0.23±0.00 ^{Db}	0.41±0.00 ^{Eb}
		14	0.24±0.00 ^{Cb}	0.43±0.00 ^{Db}
5% Na-Caseinate	No OTA	1	0.19 ±0.00^{Db}	0.33±0.04 ^{Db}
		7	0.20±0.02 ^{Db}	0.43±0.00 ^{Db}
		14	0.24±0.02 ^{Db}	0.52±0.00 ^{Db}
10% SRPH	No OTA	1	2.22±0.01 ^{Aa}	2.30±0.00 ^{Ba}
		7	2.94±0.02 ^{Aa}	6.12±0.00 ^{Aa}
		14	5.51±0.12 ^{Aa}	10.44±0.25 ^{Aa}
	1% OTA/ Pre-emulsification	1	0.35±0.00 ^{Db}	1.42±0.01 ^{Cb}
		7	1.20±0.06 ^{Ca}	2.07±0.05 ^{Cb}
		14	1.57±0.04 ^{Cb}	2.63±0.02 ^{Cb}
	1% OTA/ Post-emulsification	1	1.70±0.06 ^{Ba}	2.71±0.18 ^{Aa}
		7	2.64±0.01 ^{Ba}	3.18±0.01 ^{Bb}
		14	3.56±0.22 ^{Ba}	4.50±0.00 ^{Bb}
10% Tween 20	No OTA	1	0.75±0.05 ^{Ca}	1.07±0.02 ^{Da}
		7	0.96±0.00 ^{Da}	1.15±0.00 ^{Da}
		14	1.04±0.02 ^{Da}	1.31±0.03 ^{Da}
10% Na-Caseinate	No OTA	1	0.22±0.01 ^{Ea}	0.56±0.05 ^{Ea}
		7	0.63±0.05 ^{Ea}	0.72±0.01 ^{Ea}
		14	0.77±0.02 ^{Ea}	0.79±0.02 ^{Ea}

Values are mean ± SD (n = 5)

Different lowercase superscripts in the same column within the same emulsifier and process at the same storage time indicate significant difference ($p < 0.05$).

Different uppercase superscripts in the same column within the same level of emulsifier indicate significant difference ($p < 0.05$).

For emulsion containing 10% SRPH, the highest increase in size was observed, regardless of emulsification stage. The largest d_{32} and d_{43} was found in sample without OTA incorporation, indicating the lowest emulsion stability. For emulsion stabilized by Tween 20 and Na-caseinate, only slight increases in d_{32} and d_{43} were observed up to 14 days. When comparing between emulsion stabilized by Tween 20 or Na-caseinate at levels of 5 and 10%, emulsion containing both Tween 20 and Na-caseinate at 5% showed the lower d_{32} and d_{43}. Therefore, the concentration of surfactant or emulsifier directly affected the emulsion.

7.4.1.2 Coalescence and flocculation

Table 21 shows coalescence and flocculation of emulsions containing SRPH at various concentrations without and with OTA as affected by emulsification stage. Coalescence, a crucial factor, corresponds to the instability of the emulsion. The increase in coalescence index attributes to the collapse of the oil droplet as evidenced by the higher d_{43} (Table 20). This larger oil droplet indicates poor emulsion stability (Fredrick *et al.*, 2010). In general, the lower C_i was observed for samples with OTA incorporation ($p < 0.05$). The higher coalescence of droplets in SRPH-emulsions without OTA was presumably caused by the reduction of repulsive force between the droplets. It was noted that emulsions added with OTA were more stable. For emulsion containing 5% SRPH, C_i was higher than those containing 10% SRPH ($p < 0.05$). This was in agreement with a slight change in d_{43} (Table 20). The lower rate of coalescence was observed for emulsions containing SRPH and 1% OTA with post-emulsification. This might be due to the relatively thicker and more stable interfacial membranes, providing better steric stabilization. During the extended storage, C_i was markedly increased in all emulsion samples. It was noted that the addition of OTA with post-emulsification into emulsion containing SRPH at 10% yielded the emulsion with the highest physical stability. Nevertheless, at the same storage time, C_i of emulsion containing Tween 20 or Na-caseinate was lower than those with SRPH, regardless of OTA incorporation. This might be attributed to different hydrophobicity and conformation of different emulsifiers/surfactants.

Table 2 Effect of OTA incorporation on flocculation, coalescence of emulsion containing SRPH as affected by pre- and post-emulsification during storage of 14 days

Samples	Processes	Storage time (days)	Coalescence index (C_i)	Flocculation factor (F_f)
SRPH 5%	No OTA	1	-	1.67±0.00 ^{Aa}
		7	141.57±15.37 ^{Aa}	1.18±0.07 ^{Da}
		14	181.07±3.15 ^{Ab}	1.07±0.01 ^{Db}
	1% OTA/ Pre-emulsification	1	-	1.50±0.06 ^{Ba}
		7	103.19±1.78 ^{Bb}	1.31±0.01 ^{Ca}
		14	149.00±1.18 ^{Ba}	1.20±0.01 ^{Ca}
	1% OTA/ Post-emulsification	1	-	1.13±0.10 ^{Cb}
		7	29.33±0.10 ^{Ca}	1.13±0.00 ^{Db}
		14	76.79±1.32 ^{Ca}	1.20±0.01 ^{Cb}
Tween 5%	No OTA	1	-	1.16±0.05 ^{Ca}
		7	10.20±1.02 ^{Da}	1.57±0.01 ^{Ba}
		14	16.02±0.72 ^{Ea}	1.89±0.01 ^{Aa}
Na-Caseinate 5%	No OTA	1	-	1.23±0.16 ^{Ca}
		7	31.53±0.60 ^{Ca}	1.74±0.01 ^{Aa}
		14	57.40±0.21 ^{Da}	1.81±0.00 ^{Ba}
SRPH 10%	No OTA	1	-	1.35±0.00 ^{Bb}
		7	166.45±0.14 ^{Aa}	1.28±0.00 ^{Ba}
		14	354.90±10.94 ^{Aa}	1.21±0.03 ^{BCa}
	1% OTA/ Pre-emulsification	1	-	1.42±0.01 ^{Ba}
		7	45.99±0.00 ^{Bb}	1.17±0.03 ^{Db}
		14	85.15±1.44 ^{Bb}	1.19±0.01 ^{Ca}
	1% OTA/ Post-emulsification	1	-	1.67±0.11 ^{Aa}
		7	17.52±0.54 ^{Db}	1.5±0.01 ^{Aa}
		14	66.25±0.11 ^{Cb}	1.25±0.00 ^{Aa}
Tween 10%	No OTA	1	-	1.08±0.02 ^{Ca}
		7	7.89±0.28 ^{Eb}	1.22±0.00 ^{Cb}
		14	22.14±2.79 ^{Ea}	1.23±0.03 ^{ABb}
Na-Caseinate 10%	No OTA	1	-	1.17±0.11 ^{Ca}
		7	28.79±2.05 ^{Ca}	1.14±0.02 ^{Eb}
		14	40.21±3.21 ^{Db}	1.06±0.03 ^{Db}

Values are mean ± SD (n = 5)

Different lowercase superscripts in the same column within the same emulsifier and process at the same storage time indicate significant difference ($p < 0.05$).

Different uppercase superscripts in the same column within the same level of emulsifier indicate significant difference ($p < 0.05$).

For flocculation factor (F_f), emulsions containing 10% SRPH more likely showed the higher F_f than those containing 5% SRPH, irrespective of OTA incorporation ($p < 0.05$). This was in accordance with the higher d_{32} and d_{43} in these samples (Table 20). Self-assembly of SRPH along with bridging effects was concomitant with the increased particle size during storage (Gudipati *et al.*, 2010). For emulsion containing 5% SRPH and OTA, post-emulsification resulted in the lower F_f than pre-emulsification. On the other hand, post-emulsification caused the higher F_f than pre-emulsification when OTA was incorporated to emulsion containing 10% SRPH. During the storage of 14 days, F_f of emulsion containing SRPH without and with OTA incorporation gradually decreased. Nevertheless, emulsions containing Na-caseinate or Tween 20 had the increased F_f with increasing storage time ($p < 0.05$). Oil droplet in SRPH-emulsions containing OTA plausibly underwent flocculation at a faster rate. As a result, the coalescence could be enhanced at the higher rate as shown by the higher C_i. Higher F_f –value was concomitant with larger oil within the first 7 days of storage in all SRPH-emulsions. In general, OTA incorporation, SRPH concentrations as well as emulsification stage affected the stability of emulsion. OTA addition with post-emulsification could improve stability of emulsion particularly when 10% SRPH was used.

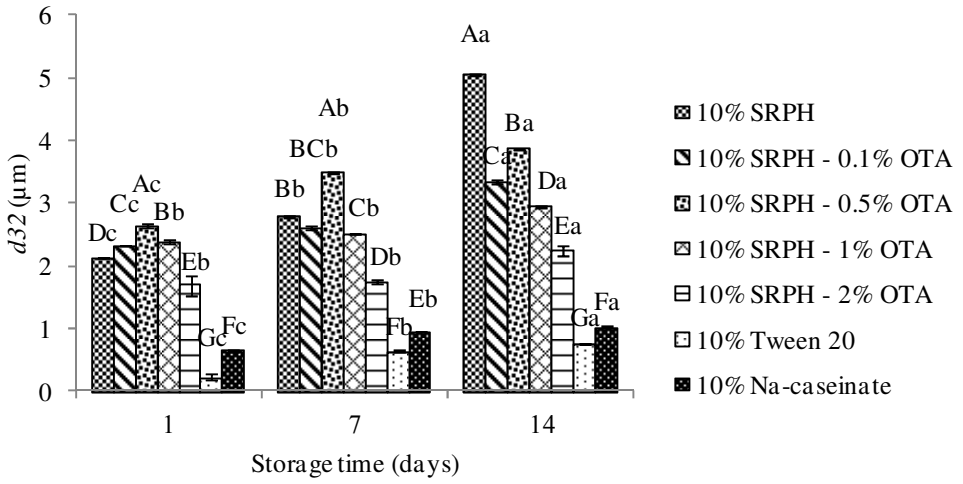
7.4.2 Stability of emulsions containing SRPH and OTA at various concentrations

Emulsions containing 10% SRPH added with OTA at various concentrations with post-emulsification underwent coalescence and flocculation differently during the storage.

7.4.2.1 Particle size

Figure 21 shows particle size of emulsions stabilized by 10% SRPH as affected by various OTA concentrations during 14 days of storage. At day 1, d_{32} (Figure 21A) and d_{43} (Figure 21B) varied with OTA concentrations used, in which both d_{32} and d_{43} increased with increasing OTA concentration up to 0.5% ($p < 0.05$). Thereafter, d_{32} and d_{43} decreased ($p < 0.05$).

(A)



(B)

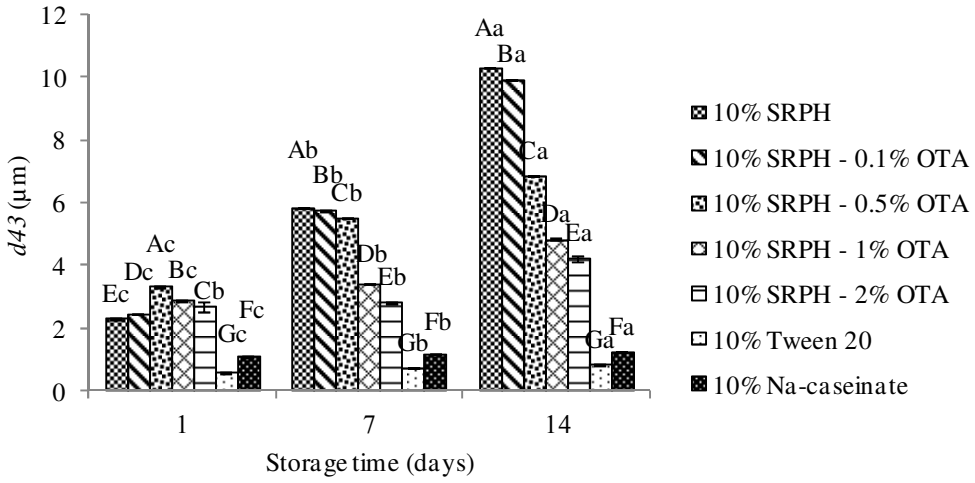


Figure 1 d_{32} (A) and d_{43} (B) of droplets in emulsions containing SRPH and OTA at different concentrations in comparison with those containing Tween 20 or Na-caseinate during storage of 14 days. Bars represent standard deviation (n=5). Different lowercase letters on the bars within the same sample indicate significant difference ($p < 0.05$). Different uppercase letters within the same storage time indicate significant difference ($p < 0.05$).

The incorporation of OTA below 1% might be insufficient to cross-link peptides surrounding oil droplets. Among all levels, OTA at 2% yielded emulsion with the

lowest d_{32} and d_{43}. However d_{32} and d_{43} of emulsions stabilized by 10% Tween 20 or 10% Na-caseinate were lower ($p < 0.05$). Upon extended storage, it was found that both d_{32} and d_{43} of emulsion without OTA drastically increased. For emulsion containing SRPH and OTA, the increased OTA concentrations could retard the increase in sizes of droplets, indicating that the emulsion was more stable when OTA at higher level was presented. The decreases in d_{32} and d_{43} of emulsion incorporated with OTA were obtained via post-emulsification. OTA at sufficient amount might preferably migrate and interact with SRPH adsorbed at the interface. Comparing with other OTA concentrations used, 2% OTA yielded SRPH-emulsion with the smallest particle size and slight changes in d_{43} and d_{32} during storage ($p < 0.05$). Stronger films stabilized by OTA surrounding oil droplet could enhance physical stability of emulsion.

7.4.2.2 Coalescence and flocculation

Coalescence and flocculation of emulsions containing SRPH as affected by different OTA concentrations were presented in Table 22. The incorporation of OTA into emulsion rendered stable emulsion against coalescence. Stability was more increased with increasing OTA concentrations as indicated by lower C_i. Notably, 2% OTA showed the superior stabilizing effect to Tween 20 or Na-caseinate at day 7 of storage ($p < 0.05$). With increasing storage time, C_i increased, whereas F_f tended to decrease in all samples, suggesting the development of larger particle (Figure 21). Among emulsions containing SRPH, the addition of 2% OTA effectively increased the emulsion physical stability but its efficacy was lower than Tween 20 or Na-caseinate. The physical stability of emulsion is governed by the balance of colloidal interactions, e.g., van der Waals attraction, electrostatic repulsion, and steric repulsion (Wooster and Augustin, 2007). The increase in steric stabilization with the attachment of a polymeric layer to the surface of the emulsion droplet is suggested for suppressing flocculation and coalescence formations (Wooster and Augustin, 2007). The increase in amount of OTA applied might enhance the formation of stronger SRPH film anchored at the interface, generating the higher steric stabilization. The addition of OTA could therefore reduce the development of droplet aggregation (C_i and F_f) to a certain extent.

Table 3 Stability of emulsions containing SRPH and OTA at various concentrations during storage of 14 days

Samples	OTA (%, w/w)	Storage time (days)	Coalescence index (C_i)	Flocculation factor (F_f)	ζ-potential (mV)
10% SRPH	-	1	-	1.35±0.01 ^{Ac}	-48.07±0.98 ^{Ea}
		7	155.24±0.74 ^{Ab}	1.24±0.01 ^{ABb}	-46.46±0.88 ^{Db}
		14	349.19±0.16 ^{Aa}	1.08±0.00 ^{Ca}	-32.67±0.69 ^{Cc}
	0.1	1	-	1.24±0.02 ^{Cc}	-49.68±0.95 ^{Da}
		7	135.129±1.22 ^{Bb}	1.15±0.01 ^{Cb}	-45.02±1.22 ^{Eb}
		14	305.44±0.81 ^{Ba}	1.04±0.02 ^{Ea}	-34.73±2.26 ^{Cc}
	0.5	1	-	1.09±0.01 ^{Ec}	-51.48±1.94 ^{Ca}
		7	64.98±0.12 ^{Cb}	1.26±0.00 ^{Aa}	-48.77±1.29 ^{Cb}
		14	104.97±0.06 ^{Ca}	1.16±0.00 ^{Bb}	-37.57±0.73 ^{Bc}
	1.0	1	-	1.26±0.01 ^{Cc}	-52.38±0.49 ^{Ca}
		7	17.79±0.02 ^{Eb}	1.13±0.00 ^{Db}	-47.02±1.82 ^{Db}
		14	67.23±1.00 ^{Da}	1.02±0.01 ^{Fa}	-38.47±1.12 ^{Bc}
	2.0	1	-	1.15±0.00 ^{Dc}	-59.51±0.46 ^{Ba}
		7	4.97±1.23 ^{Gb}	1.13±0.00 ^{Db}	-51.75±0.62 ^{Bb}
		14	57.14±3.32 ^{Ea}	1.06±0.00 ^{Da}	-50.04±0.95 ^{Ac}
10% Tween 20	-	1	-	1.16±0.03 ^{Dc}	-36.40±1.26 ^{Fa}
		7	28.03±2.31 ^{Db}	1.09±0.02 ^{Eb}	-25.66±0.47 ^{Fb}
		14	46.98±0.82 ^{Fa}	1.02±0.01 ^{Fa}	-25.37±3.48 ^{Db}
10% Na-Caseinate	-	1	-	1.32±0.01 ^{Ba}	-65.15±0.51 ^{Aa}
		7	7.83±0.11 ^{Fb}	1.23±0.01 ^{Bc}	-61.99±0.77 ^{Ab}
		14	15.73±1.05 ^{Ga}	1.30±0.00 ^{Ab}	-51.43±1.38 ^{Ac}

Values are mean ± SD (n = 5)

Different lowercase superscripts in the same column within the same level of OTA indicate significant difference ($p < 0.05$).

Different uppercase superscripts in the same column indicate significant difference ($p < 0.05$).

7.4.2.3 ζ-potential

Electrical charge on oil droplets of SRPH-emulsions incorporated with OTA at different concentrations was measured (Table 22). In the absence of OTA, SRPH-emulsion had ζ-potential about -48.07 mV at pH 9.0. With increasing concentrations of OTA, SRPH-emulsions had the increasing negative charge ((-49.68)-(-59.51) mV) ($p < 0.05$). At pH 9, some carboxyl groups of peptide became deprotonated as evidenced by the negative electrical charge. When OTA was added, it

might attach with some positive charged amino acid residue or induced inter-/intra molecular cross-linking, in which negative charged residues were masked. During the extended storage, all samples became less negatively charged ($p < 0.05$). The adsorbed proteins surrounding droplets plausibly underwent aggregation via ionic interaction as indicated by the change of ζ-potential. This might cause the decreased repulsion between oil droplets. Nevertheless, it was noted that SRPH-emulsion incorporated with 2% OTA could retain ζ-potential above -50 mV, which was not different from that of Na-caseinate-emulsion. ζ-potential value is generally related with the electrostatic repulsion between the droplets in emulsions. Emulsions exhibiting the absolute ζ-potential higher than +30 mV or lower than −30 mV tend to be electrostatically stable, while those having the range of (-30) - 30 mV tend to coagulate or flocculate (Wang *et al.*, 2011c). However, emulsion stabilized by Tween 20 had ζ-potential of -36.40 mV and then negatively charge reduced to -25 mV after 7 days of storage. Tween 20, a non-ionic surfactant, stabilized emulsion via incorporating hydrophobic tail into oil and forming a protective membrane surrounding oil droplet. Hence the reduction of negative charges might be governed by the changes in net charge of polar lipids in fish oil, especially phospholipids. This was coincidental with the increases in d_{32} and d_{43} (Figure 21). These results were in agreement with coalescence and flocculation (Table 22), in which OTA at 2% could increase the negative charge of peptides film, thereby lowering coalescence of emulsion.

7.4.2.4 CLSM micrograph

Particle distribution of emulsions stabilized by SRPH in the presence of OTA at different concentrations was visualized by CLSM (Figure 22). At the first day of storage, CLSM images depicted the particulate clusters or clumps of oil droplets (red color) in SRPH-emulsions. It was noted that the smaller droplet size was obtained in SRPH-emulsion incorporated with higher OTA concentrations. However, all SRPH-emulsions had higher droplet size, compared to those stabilized by Tween 20 or Na-caseinate. At day 14 of storage, the flocculation and coalescence took place in all SRPH-emulsions, particularly those without OTA.

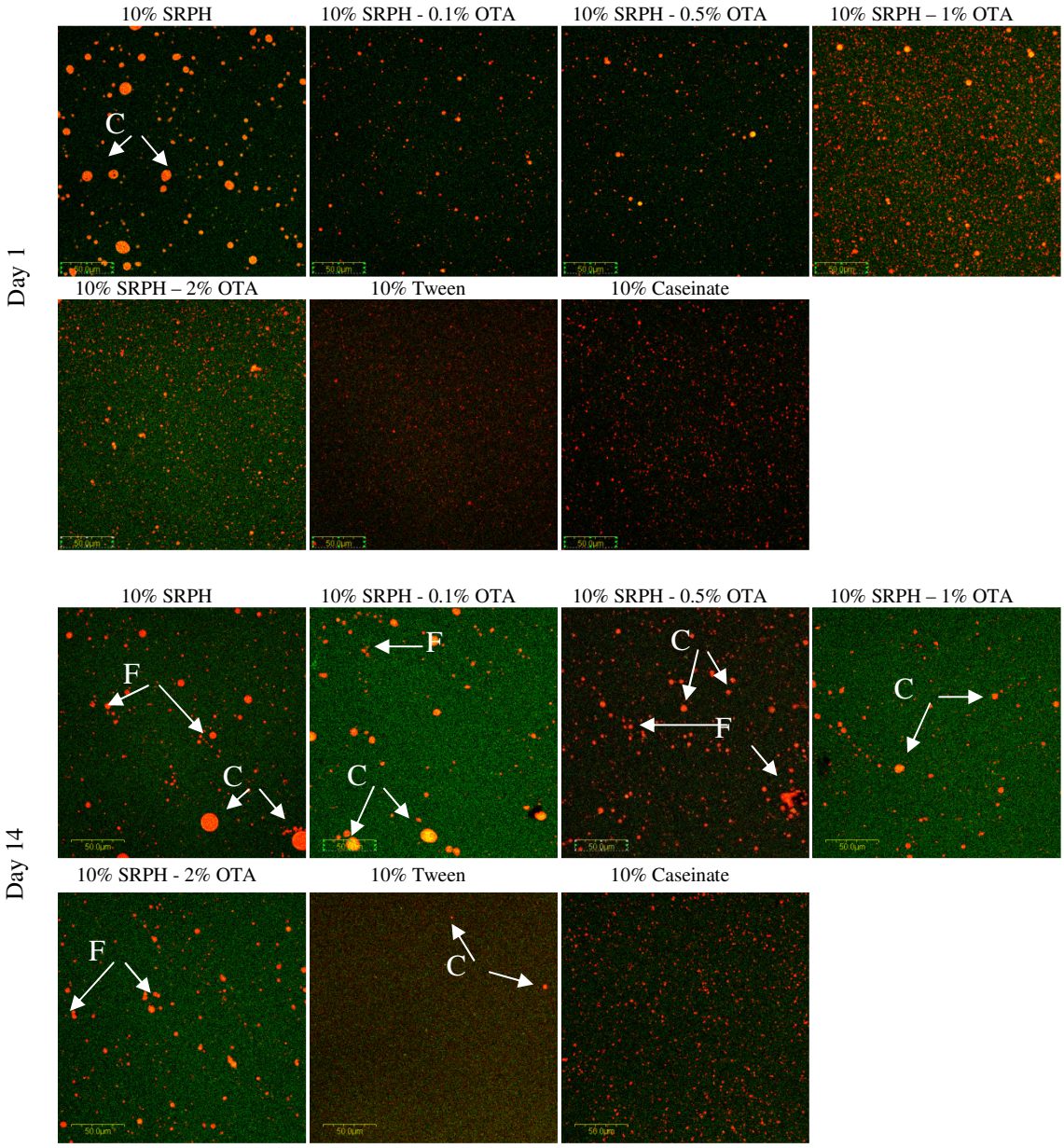


Figure 2 CLSM micrograph of emulsions containing SRPH and OTA at different concentrations in comparison with those containing Tween 20 or Na-caseinate during storage of 14 days. Magnification; 600x. Red represent lipid. F; flocculated droplet, C; coalesced droplet

Disruption of emulsion was in accordance with the increases in d_{32} and d_{43} (Figure 2), when storage time increased. No flocculation and coalescence were observed in emulsion stabilized by Tween 20 or Na-caseinate. Thus, OTA at 2% in conjunction with post-emulsification could improve the physical stability of SRPH-emulsion.

7.4.2.5 Protein pattern

Patterns of total proteins and adsorbed proteins under reducing and non-reducing condition are shown in Figure 23A and Figure 23B, respectively. Total protein and adsorbed protein patterns in emulsion stabilized by SRPH and Na-caseinate were different. For total proteins in emulsion containing SRPH, proteins with MW less than 14 kDa were dominant (Figure 23A). As OTA concentrations increased, smear band with MW above 14 kDa was observed. Coincidentally, peptides with MW less than 14 kDa were slightly decreased. Emulsion containing Na-caseinate had major protein with MW of 30 kDa. Under reducing condition, some protein bands were regained, suggesting the presence of disulfide bond when OTA was added into emulsions.

For adsorbed proteins, those with MW less than 14 kDa was the major adsorbed protein at the interface (Figure 23B). The addition of OTA with post-emulsification resulted in the appearance of protein band with higher MW. The band with increasing MW was found as OTA concentrations increased. OTA, as a cross-linking agent, induced cross-linking of peptides in SRPH localized at the interface via several bonding including disulfide bond. The protein at the oil-water interface was the crucial factor in the stability of the emulsions because proteins can lower the interfacial tension at the oil-water interface (Khan *et al.*, 2014). Taherian *et al* (2011) suggested that conformational change of adsorbed protein was related with surface hydrophobicity, protein flexibility, solubility, bondings and forces. Oxidized phenolic compounds were reported to induce the formation of disulfide bond, apart from covalent bond (Balange and Benjakul, 2009). The result suggested that OTA at higher concentrations could induce the cross-linking of SRPH films, thereby strengthening the film with concomitantly increased physical stability.

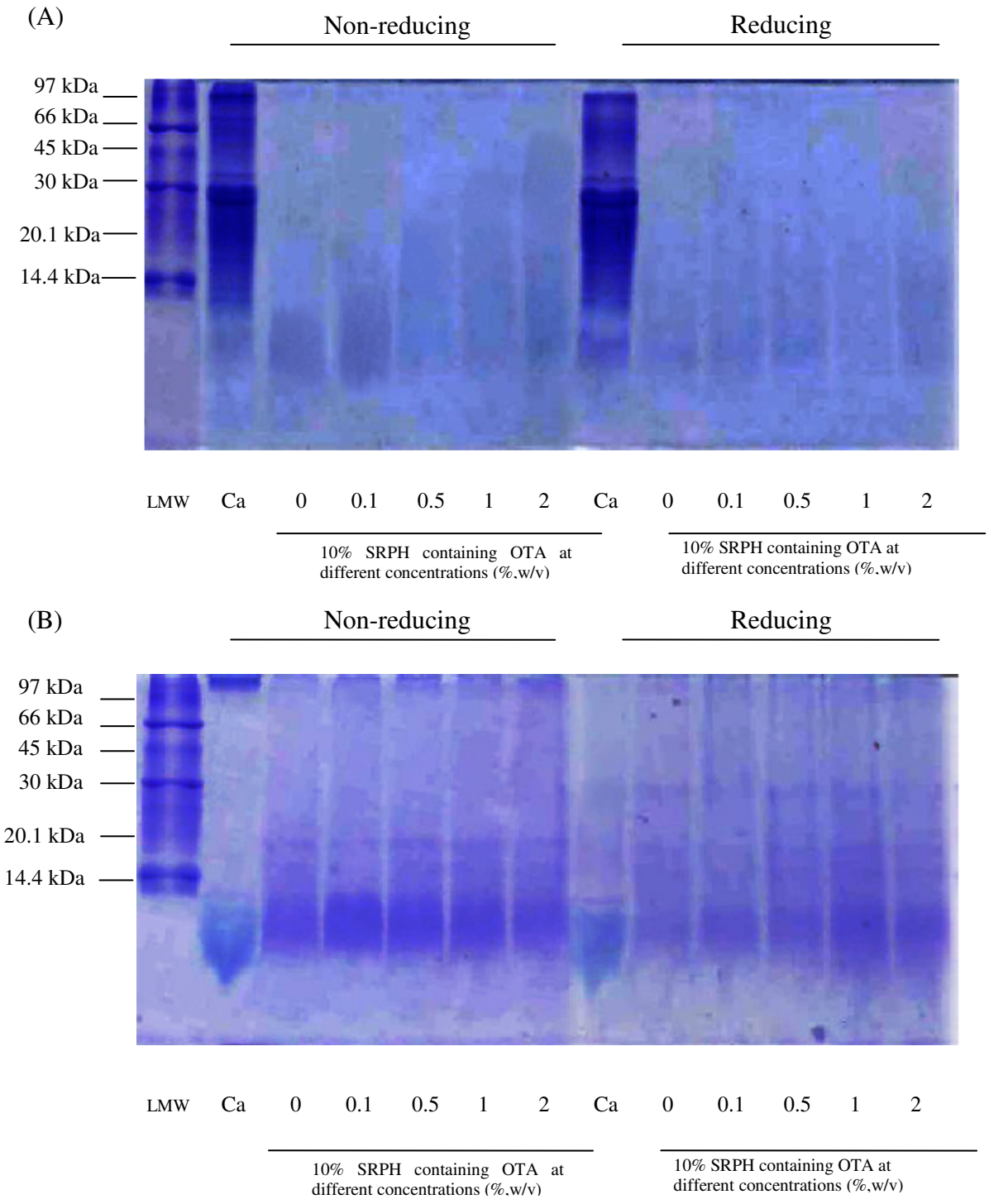


Figure 3 Electrophoretic patterns of total protein (A) and adsorbed protein (B) of emulsions containing SRPH and OTA at different concentrations in comparison with that containing Na-caseinate under reducing and non-reducing conditions. LMW: Low molecular weight marker.

7.4.2.6 Oxidation stability

Lipid oxidation of menhaden oil-in-water emulsion stabilized by SRPH without and with OTA at different concentrations was monitored and expressed as TBARS values during 14 days of storage (Figure 24). TBARS of emulsion samples gradually increased as storage time increased ($p < 0.05$). However, the rate of increase varied. The highest TBARS formation was observed for emulsion stabilized by 10% Tween 20 during 7-14 days of storage ($p < 0.05$). There was no difference in TBARS between the emulsion containing SRPH and that with Tween 20 within the first seven days of storage ($p > 0.05$). Above critical micelle concentration (2.8×10^{-5} M) (Christov *et al.*, 2002), Tween 20 might form micelle and more likely partitioned into aqueous phase. This resulted in the lower amount of Tween 20 at the interface. As a consequence, the droplets were more prone to oxidation as indicated by higher TBARS formation (McClements and Decker, 2000). The oxidative stability of emulsions containing SRPH might be due to antioxidative properties of SRPH (Intarasirisawat *et al.*, 2012). When OTA concentrations increased, the lower TBARS formation of SRPH-emulsions was observed. At the first day of storage, the addition of OTA could reduce the formation of TBARS approximately 50%, when compared to emulsion stabilized by Tween 20 or SRPH. After 14 days of storage, TBARS value was 9.88 mg MDA/ L emulsion for SRPH-emulsion containing 0.1% OTA. It was noted that the addition of 0.5% OTA could effectively inhibit the formation of TBARS and the efficacy was comparable to 1 and 2% OTA during 14 days of storage. This was evidenced by no marked changes in TBARS of samples containing 0.5 – 2% OTA throughout the storage ($p > 0.05$). Among all samples, that containing Na-caseinate had the lowest TBARS throughout the storage of 14 days ($p < 0.05$). This might be attributed by the continuous and uniform casein film surrounding droplet, which could prevent oil from oxygen. This was coincidental with the high stability of emulsion stabilized by Na-caseinate (Figure 21 and Table 22). The emulsion stabilized by SRPH in the presence of OTA had oxidative stability comparable to that stabilized by 1% gelatin modified by 5% OTA (Aewsiri *et al.*, 2009). Particle size, surface area, charge, availability of transition metals and thickness of interfacial region had the impact on oxidative stability of emulsions (Gudipati *et al.*, 2010). The

emulsion droplet size can influence lipid oxidation rates because smaller particle sizes are related with a larger surface area for oxidation. Therefore, emulsion stabilized by Tween 20 possessing the smallest particle size and the largest surface area had the highest TBARS values. Phenolic compounds interacted with protein was reported for better retardation of lipid oxidation than free form since free phenolics were preferably localized in the aqueous phase (Aewsiri *et al.*, 2009; Aewsiri *et al.*, 2013; Almajano and Gordon, 2004). Lipid oxidation in oil-in-water emulsion commonly occurs at the interface. SRPH-OTA complex localized at the interface was assumed to prevent lipid oxidation (Yuji *et al.*, 2007). The addition of OTA to SRPH-emulsions was presumable to form thick layer surrounding the oil droplet, thereby preventing the penetration of oxygen into oil droplet more effectively (Aewsiri *et al.*, 2009). Oxidative stability of SRPH-emulsion could be improved by addition of OTA at least with the concentration of 1%.

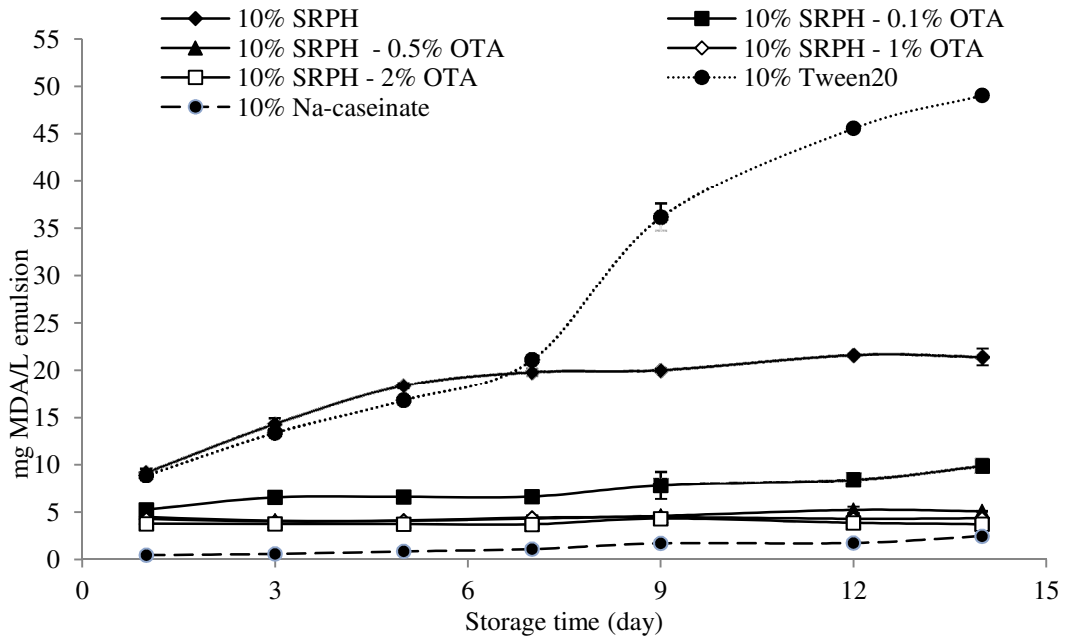


Figure 4 TBARS values of menhaden oil-in-water emulsions containing SRPH without and with OTA at various concentrations in comparison with those containing Tween 20 or Na-caseinate during storage at 30 °C for 14 days. Bars represent the standard deviation (n=3).

7.5 Conclusion

Addition of OTA into emulsion containing SRPH as emulsifier could retard the coalescence. Post-emulsification yielded emulsion containing both SRPH and OTA with increased stability. The stability of emulsion was also affected by OTA concentration. The addition of OTA at least 1% was able to enhance the oxidative stability of menhaden oil-in-water emulsion.

CHAPTER 8

Microencapsulation and stability of fish oil as affected by skipjack roe protein hydrolysate and tannic acid

8.1 Abstract

Microencapsulation of fish oil was achieved by spray drying the micro-emulsion, in which skipjack roe protein hydrolysate (SRPH: degree of hydrolysis, 5%) was used as the wall material in the absence or presence of tannic acid (TA) or oxidized tannic acid (OTA). Microcapsule using SRPH had the lower encapsulation efficiency (EE) than those having sodium caseinate-whey protein concentrate (Ca-WPC) as the wall materials ($p < 0.05$). The incorporation of TA or OTA in combination with SRPH yielded spherical encapsulated fish oil with higher EE but lower particle size ($p < 0.05$), than the use of SRPH alone. Furthermore, the addition of TA could retard lipid oxidation of microcapsule more effectively as indicated by lower peroxide value and TBARS value during 4 weeks of storage, compared with those using only SRPH ($p < 0.05$). Nevertheless, OTA in conjunction with SRPH yielded the higher EE than TA. Therefore, SRPH could serve as an alternative wall material for microencapsulation of fish oil, especially with the aid of TA or OTA.

8.2 Introduction

Fish oil is considered as an abundant source of polyunsaturated fatty acids (PUFAs), particularly docosahaxaenoic acid (DHA) and eicosapentaenoic acid (EPA). Those PUFAs have been known to positively be of health benefit. The U.K. Department of Health and European Academy of Nutritional Science recommend a daily intake of 200 mg EPA and DHA (Garg *et al.*, 2006). Despite high nutritive value, the use of fish oil in food products is limited owing to the high susceptibility of PUFAs to oxidation. The development of fishy and metallic off-flavors takes place in fish oil and their emulsion, thereby lowering the acceptability. To prevent the deterioration caused by oxidation, microencapsulation has been introduced by protecting core ingredients from environment, lowering flavor loss, contributing to maintain mouthfeel perception and prolonging shelf-life of product

(Klaypradit and Huang, 2008). Spray drying is a popular technique for encapsulation of lipophilic food ingredients, due to cost-effectiveness, flexibility and high potential capacity to produce particles of good quality. Recently, several studies have been undertaken to encapsulate fish oil via spray drying using different wall materials (Aghbashlo *et al.*, 2013b) and drying conditions (Aghbashlo *et al.*, 2013b; Kagami *et al.*, 2003).

Proteins from egg yolk including lipovitellin and phosvitin have been demonstrated as the potential emulsifiers (Daimer and Kulozik, 2009). Additionally, its hydrolysate such as skipjack roe protein hydrolysate was reported to possess antioxidative activity (Intarasirisawat *et al.*, 2012). Nevertheless, skipjack roe protein hydrolysate (SRPH) having 5% DH showed low emulsifying properties. High pressure homogenization and the incorporation of OTA could improve the stability of emulsion containing SRPH with 5% DH (Intarasirisawat *et al*., 2014). Generally, tannic acid could function as the potential natural antioxidant for controlling oxidation of fish oil-in-water emulsion (Aewsiri *et al.*, 2009; Maqsood and Benjakul, 2010). Casein hydrolysate was used as wall material for fish oil encapsulation (Molina Ortiz *et al.*, 2009). Nevertheless, there is no information on the use of fish protein hydrolysate as a wall material for micro-encapsulated oil. Moreover, the addition of non-oxidized or oxidized tannic acid could improve the property of wall and enhance the oxidative stability of encapsulated fish oil. Thus, this study aimed to investigate the effect of microencapsulation of fish oil using SRPH as wall material in the absence or presence of tannic acid with various forms.

8.3 Material and methods

8.3.1 Chemicals

Menhaden oil, tannic acid (TA), sodium azide and sodium caseinate (SC) were obtained from Sigma Chemical Co. (St. Louis, MO, USA). Trichloroacetic acid (TCA), 2-thiobarbituric acid (TBA), ferric chloride, methanol, 1,1,3,3-tetramethoxypropane and sodium sulfite were purchased from Merck (Darmstadt, Germany). Hexane and isopropanol were obtained from ACI Lab-Scan (Bangkok,

Thailand). Whey protein concentrate (WPC) was procured from I.P.S. International Co., Ltd. (Bangkok, Thailand). All chemicals were of analytical grade.

8.3.2 Preparation of oxidized tannic acid

Oxidized tannic acid (OTA) was prepared according to the method of Aewsiri *et al.* (2009). Tannic acid (2%, w/v) was dissolved in distilled water, followed by a pH adjustment to 9 using 1 M NaOH. Solution was then bubbled with high purity oxygen (99. 5%) (Thai Industrial Gases PCL, Songkhla, Thailand) at 40 °C for 1 h for conversion of tannic acid to OTA.

8.3.3 Preparation of SRPH

SRPH was prepared as per the method of Intarasirisawat *et al.* (2012) to obtain a degree of hydrolysis (DH) of 5%. Briefly, the defatted roe was suspended in distilled water to obtain protein concentration of 20 mg/mL. The mixture was homogenized and pre-incubated at 50 °C for 20 min. The hydrolysis reaction was initiated by addition of Alcalase (0.15 unit/ g protein). The reaction was conducted at pH 8 and 50 °C for 1 h. The enzymatic reaction was terminated by heating at 85 °C for 15 min. DH of the resulting hydrolysate was measured as per the method of Benjakul and Morrissey (1997). The obtained skipjack roe protein hydrolysate (SRPH) was lyophilized using a freeze-dryer (CoolSafe 55, ScanLaf A/S, Lynge, Denmark), placed in polyethylene bag and kept at -20 °C until use.

8.3.4 Microencapsulation of fish oil

8.3.4.1 Preparation of emulsion

Twenty-four grams of SRPH were suspended in distilled water. The pH of SRPH solution was adjusted to 9 using 1 M NaOH. SRPH solution (70 mL; solid 345-350 mg/mL) was added with 6 mL of menhaden oil to obtain 30% total solid. The mixture was then emulsified at a speed of 10,000 rpm for 2 min using a homogenizer (Model T25 basic, IKA LABORTECHNIK, Selangor, Malaysia). The coarse emulsions were then passed through a Microfluidics homogenizer (Model HC-

5000, Microfluidizer, Newton, MA, USA) at pressure of 2,000 psi for thirteen passes. Thereafter, TA or OTA at a level of 2% (w/v) was added and the mixtures were additionally homogenized for two passes. The obtained emulsions were referred to as 'SRPH-TA' and 'SRPH-OTA', respectively. The control termed 'SRPH' was prepared in the same manner except TA or OTA was excluded. Emulsion containing sodium caseinate (Ca) and whey protein concentrate (WPC) at a ratio of 1:1 termed 'Ca-WPC' was also prepared and used as the positive control. NaN_3 (0.02% of total sample) was added to the emulsions as an antimicrobial agent. The obtained emulsions were subsequently spray-dried.

8.3.4.2 Spray drying

Spray drying was performed using a laboratory scale spray dryer LabPlant SD-05 (Huddersfield, England) with a spray chamber (500 mm × 215 mm) and a nozzle (1.5 mm diameter). The emulsion was fed into the main chamber through a peristaltic pump using a feed flow rate of 5 mL/min. Drying air flow rate was 4.3 m/s and compressor air pressure was 0.06 MPa. Air inlet temperature was applied at 200±2 °C. The outlet temperature was controlled at 108±2 °C. All encapsulated samples were subjected to analyses.

8.3.5 Analyses

8.3.5.1 Moisture content and color

Moisture content was determined by drying the sample in a vacuum oven (Memmert, Schwabach, Germany) at 70 °C for 24 h (AOAC, 1984).

The color of sample was determined using a colorimeter (ColorFlex, Hunter Lab Reston, VA, USA) and reported in the CIE system including L^*, a^* and b^* and ΔE^*, representing lightness, redness/greenness, yellowness/blueness and total difference of color, respectively. The ΔE^* was 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 ($L^* = 93.55$, $a^* = -0.84$, $b^* = 0.37$)

8.3.5.2 Powder size

Powder size distribution was measured using a laser light diffraction instrument, Mastersizer (Laser Scattering Spectrometer Mastersizer model MAM 5005, Malvern Instruments Ltd., Worcestershire, UK) according to the method of Carneiro *et al.* (2013). Briefly, a small sample was suspended in isopropanol and the particle size distribution was monitored during each measurement until successive readings became constant. The particle size was expressed as d_{43}, the volume weighted mean diameter, which is the mean diameter of a sphere with the same volume, and is generally used to characterize a particle (Jafari *et al.*, 2008a).

8.3.5.3 Powder morphology

Powder morphology was evaluated by a scanning electron microscopy (SEM). Powders were mounted on a bronze stub and sputter-coated with gold (Sputter coater SPI-Module, West Chester, PA, USA). The specimens were observed using a scanning electron microscope (Quanta 400, FEI, Eindhoven, Netherlands) at an acceleration voltage of 15 kV with magnification of 3000×.

8.3.5.4 Encapsulation efficiency (EE)

To determine the surface oil, 15 mL of hexane were added into 2 g of powder. The mixture was shaken with a vortex mixer (Vortex-genie2, G-560E, NY, USA) for 2 min at room temperature. The solvent mixture was then filtered through a Whatman no. 1 filter paper (Whatman International Ltd., Maidstone, England) and the collected powder on the filter was rinsed three times with 20 mL of hexane (Bae and Lee, 2008). The filtrate containing the extracted oil was transferred to a clean flask, which was left to evaporate and then was dried at 60 °C until constant weight was obtained. The surface oil was calculated based on the difference between the initial clean flask and that containing the oil extracted from the surface (Jafari *et al.*, 2008a).

Total oil was determined using the method described by Shahidi and Wanasundara (1995). Five gram of powder was dissolved in 25 mL of 0.88% (w/v) KCl solution. Then 50 mL of chloroform and 25 mL of methanol were added. The

mixture was then homogenized using a homogenizer for 5 min at 9,500 rpm. The mixture was transferred to a separation funnel; the chloroform layer was separated and then evaporated using a rotary evaporator at 60 °C to recover the oil. The encapsulation efficiency (EE) was calculated by the following equation:

$$EE\ (\%) = (W_{total\ oil} - W_{surface\ oil})/W_{total\ oil} \times 100$$

where $W_{total\ oil}$ represents the weight of oil added initially in the particle formation mixture. $W_{surface\ oil}$ represents the weight of surface oil.

8.3.5.5 Oxidative stability

Different encapsulated menhaden oil samples were stored in the amber bottle and capped tightly. The samples were stored at 30 ± 1 °C and taken for analyses every week for up to 4 weeks.

A. Peroxide value (PV)

PV was determined according to the method of Takeungwongtrakul and Benjakul (2013). The powder (0.5 g) was dissolved in 5 mL of distilled water. Then the mixture was mixed using a vortex mixer for 5 min to allow complete dispersion. To 1 mL of the mixture, 2 mL of chloroform/methanol (2:1, v/v) were added and mixed using a vortex mixer for 3 s to separate the sample into two phases. The organic solvent phase (20 µL) was mixed with 2.35 mL of chloroform/methanol (2:1, v/v), followed by 50 µL of 30% ammonium thiocyanate (w/v) and 50 µL of 20 mM ferrous chloride solution in 3.5% HCl (w/v). After 20 min, the absorbance of the colored solution was read at 500 nm using a spectrophotometer (Shimadzu, Kyoto, Japan). Blank was prepared in the same manner, except the distilled water was used instead of ferrous chloride. PV was calculated after blank substraction and expressed as mg cumene hydroperoxide/g oil. A standard curve was prepared using cumene hydroperoxide with the concentration range of 0.5–2 ppm.

B. Thiobarbituric acid reactive substances (TBARS)

TBARS were determined as described by Buege and Aust (1978). Powder sample (0.5 g) was dispersed in 5 mL of distilled water. Then 0.5 mL of the mixture was mixed with 2.5 mL of a solution containing 0.375% thiobarbituric acid (w/v), 15% trichloroacetic acid (w/v) and 0.25 M HCl. The mixture was heated in a boiling water (95-100 °C) for 10 min to develop a pink color, cooled with running tap water and centrifuged at 3600x*g* at 25 °C for 20 min using a centrifuge (Hettich mikro 20, Tuttlingen, Germany). The absorbance of the supernatant was measured at 532 nm using a spectrophotometer. A standard curve was prepared using 1,1,3,3-tetramethoxypropane at the concentrations ranging from 0 to 6 ppm. TBARS were calculated and expressed as mg malonaldehyde kg/sample.

8.3.6 Statistical analysis

All experiments were run in triplicate using different three lots of samples. Statistical analysis was performed using one-way analysis of variance (ANOVA). Mean comparison was carried out using Duncan's multiple range test. (Steel and Torrie, 1980). Analysis was performed using the SPSS package (SPSS for windows, SPSS Inc., Chicago, IL, USA).

8.4 Results and discussion

8.4.1 Characteristics of fish oil microcapsules

8.4.1.1 Moisture content

The characteristics of the microcapsules prepared using different wall materials are displayed in Table 23. Moisture content of fish oil microcapsules ranged from 1.13 to 3.10% (w/w). The lowest moisture content was observed for Ca-WPC sample (1.13%) ($p < 0.05$). This might be due to hydrophobic nature of Ca and WPC. As a consequence, the reduced absorption and permeation of water could be obtained (Wang *et al.*, 2011b). SRPH had more hydrophilicity, associated with increasing charged residues, e.g. carboxyl or amino groups.

Table 1 Moisture content and some characteristics of fish oil microcapsules prepared using SRPH without and with TA or OTA as wall material

Properties	SRPH	SRPH-TA	SRPH-OTA	Ca-WPC
Moisture content (%)	1.66±0.09 [†C‡]	3.10±0.18 [A]	2.04±0.04 [B]	1.13±0.11 [D]
Color				
*L	84.59±0.43 [C]	88.08±0.05 [B]	80.04±0.02 [D]	95.56±0.02 [A]
*a	0.31±0.06 [D]	1.69±0.07 [A]	1.30±0.04 [B]	0.84±0.09 [C]
*b	23.22±0.11 [A]	15.83±0.04 [C]	21.53±0.06 [B]	11.22±0.16 [D]
Δ*E	24.67±0.08 [B]	16.46±0.02 [C]	25.12±0.05 [A]	11.06±0.15 [D]
Powder size (μm)	17.07±0.57 [A]	6.16±0.09 [C]	6.37±0.08 [C]	10.23±0.10 [B]

[†] Values are mean ± SD (n=3)

[‡]Different superscripts within the same row indicate significant difference ($p < 0.05$).

The enzymatic hydroylsis provided hydrolysate with more exposed hydrophilic groups (Chabanon *et al.*, 2007). When tannic acid was incorporated, the increase in moisture content was found for microcapsules (SRPH-TA). Tannic acid possessing high number of hydroxyl groups might favor water adsorption via hydrogen bonding. For SRPH-OTA sample, the lower moisture content was found, in comparison with SRPH-TA counterpart. With lower amount of available hydroxyl groups, OTA in wall material more likely had the lower ability to adsorb water. In the present study, the inlet drying temperature was 200 °C. Inlet drying temperature had the impact on moisture content of the encapsulated fish oil (Aghbashlo *et al.*, 2013b; Tonon *et al.*, 2011). With increasing inlet drying temperature (from 140 to 180 °C), moisture content decreased significantly, regardless of wall materials used (Aghbashlo *et al.*, 2013b). Moisture content of the resulting microcapsule observed in the present study was lower than that of fish oil powder coated with rice bran fiber (5.92%) (Wan *et al.*, 2011) or n-octenylsuccinate-derivatized starch and glucose syrup DE10 (3.65%) (Drusch *et al.*, 2006). Dry power with moisture contents range of 3-10% has a good

stability during storage (Klaypradit and Huang, 2008; Quispe-Condori *et al.*, 2011). The moisture content and water activity correlate with appearance, oxidation stability and flowability of the obtained microcapsules (Drusch *et al.*, 2006). In general, powder possessing high moisture content has sticky appearance and low flowability (Bae and Lee, 2008). When surface droplet viscosity and powder particle stickiness increased, inter-particle bridge formation takes place. Moisture content in combination with storage temperature affect the shelf-life of dried microcapsules (Drusch *et al.*, 2006). Thus, moisture content of the resulting microcapsules was governed by wall material used as well as form of tannic acid.

8.4.1.2 Color

Color of different fish oil microcapsules is presented in Table 23. Lightness of all SRPH-samples was lower than that of Ca-WPC ($p < 0.05$). For SRPH samples, the addition of TA rendered microcapsules with the higher lightness. On the other hand, the incorporation of OTA resulted in the decreased L*-value ($p < 0.05$). The addition of TA or OTA also increased the redness (*a) but decreased the yellowness (*b). The darker color of SRPH-OTA microcapsule might be due to the quinone in OTA. Among all samples, SRPH-OTA microcapsule had the highest total color difference (ΔE^*), followed by SRPH, SRPH-TA and Ca-WPC microcapsules, respectively. As a result, the color of the microcapsule was determined by the form of the TA used as well as the type of wall material.

8.4.1.3 Particle size

The particle size distribution of spray- dried microcapsules is shown in Table 23. Particle mean diameters varied from 6.16 to 17.07 µm. The highest particle size was observed for SRPH-microcapsules ($p < 0.05$). Microcapsules consisting of biodegradable polymers such as protein, carbohydrate, hydrocolloids, etc. have ideally particle size less than 200 µm (Venkatesan *et al.*, 2009). The diameter of the microcapsules depends probably on homogenization parameters, the materials used and spray drying condition (Kolanowski *et al.*, 2005). Additionally, particle size is influenced by the inlet drying temperature (Aghbashlo *et al.*, 2013b). Larger particle

size could be obtained by drying at the higher temperature. The faster drying rate accelerates the water migration of crust forming soluble materials to the surface of atomized droplets (Aghbashlo *et al.*, 2013b). The particle size of SRPH-microcapsules was higher than fish oil encapsulated by casein hydrolysate (9.18-11.32 μm) (Molina Ortiz *et al.*, 2009). It was noted that the particle size of microcapsules decreased as TA or OTA was incorporated ($p < 0.05$), in comparison with that of SRPH sample. This was plausibly associated with the increased emulsion stability regulated by TA or OTA (Intarasirisawat *et al*., 2014). As a result, the coalescence became lowered in the presence of TA or OTA. After drying, those small droplets turned to powder with smaller particle size. Powder size of microcapsule using SRPH in combination with TA or OTA was lower than that of microcapsule with Ca-WPC as wall material ($p < 0.05$). Thus, the use of SRPH along with TA or OTA as wall material yielded the microcapsules with smaller size, in which Ca-WPC could be replaced.

8.4.1.4 EE

EE of the microcapsule as affected by different wall materials is shown in Figure 25. The lowest EE was observed for SRPH sample (13%), followed by SRPH-TA (28%), SRPH-OTA (55%) and Ca-WPC (70%), respectively. The improved EE was found in the microcapsule prepared using SRPH-OTA, compared with SRPH or SRPH-TA ($p < 0.05$). OTA, as a cross-linking agent, might induce cross-linking of peptides in SRPH localized at the interface via several bonding including disulfide bond (Intarasirisawat *et al*., 2014). The higher EE of Ca-WPC microcapsule could be attributed to the higher emulsion viscosity, thereby impeding the oil diffusion from the core of the spray-dried droplets to the surface (Aghbashlo *et al.*, 2013b). The low EE indicated the higher amount of oil, which could not trap in the matrix of the microcapsule called ‘surface oil’. The surface oil is susceptible to oxidation than the encapsulated oil. The retention of these cores is governed by their chemical functionality, solubility, polarity and volatility (Gharsallaoui *et al.*, 2007). EE observed in the present study was lower than those reported for fish oil encapsulated by different proteins such as barley protein (92.9-100%) (Wang *et al.*, 2011b), pea protein isolate and maltodextrin (83.34%) (Aberkane *et al.*, 2013) and

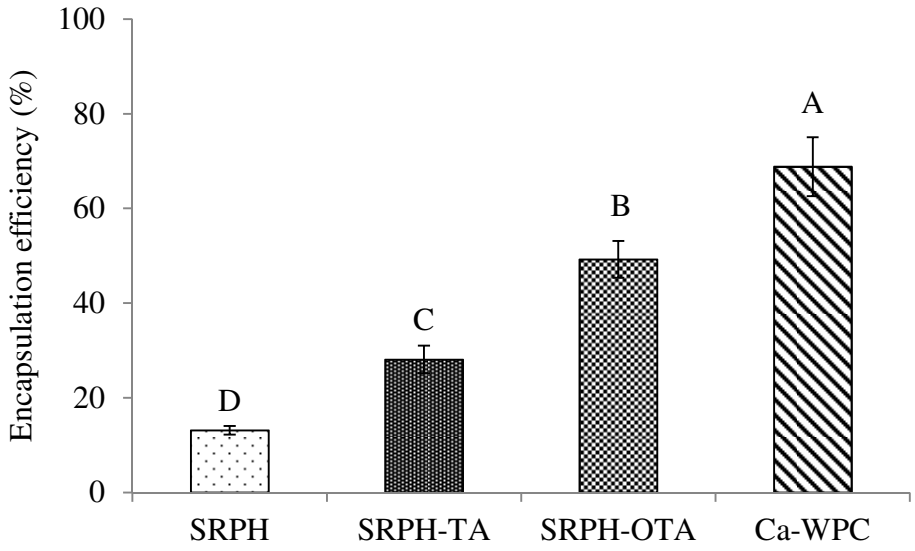


Figure 1 Encapsulation efficiency of fish oil prepared using SRPH without and with TA or OTA as wall materials. Different uppercase letters on the bars denote significant difference ($p < 0.05$). Bars represent the standard deviation (n=3).

casein and maltodextrin (83.54%) (Klaypradit and Huang, 2008). The lower EE of all SRPH samples might be due to short chain peptide in SRPH, which more likely formed the weaker film surrounding oil droplet. Additionally, EE of samples was significantly influenced by the type of coating material used, which have both different retention properties and film-forming capacity (Aberkane *et al.*, 2013). The ability of the encapsulant to form a good emulsion before drying, a prerequisite for high encapsulation efficiency, depends on the inherent film forming properties of the encapsulant mixture (Augustin *et al.*, 2006). The results suggested that the addition of OTA could improve EE of the microcapsule using SRPH as the wall material.

8.4.1.5 Morphology

Microstructure of fish oil microcapsules with different wall materials varied (Figure 26). Some pores were noticeable in the microcapsule prepared using SRPH as wall material (Figure 26A). This could facilitate the permeation of oxygen, which subsequently induced the deterioration of lipid, a core material.

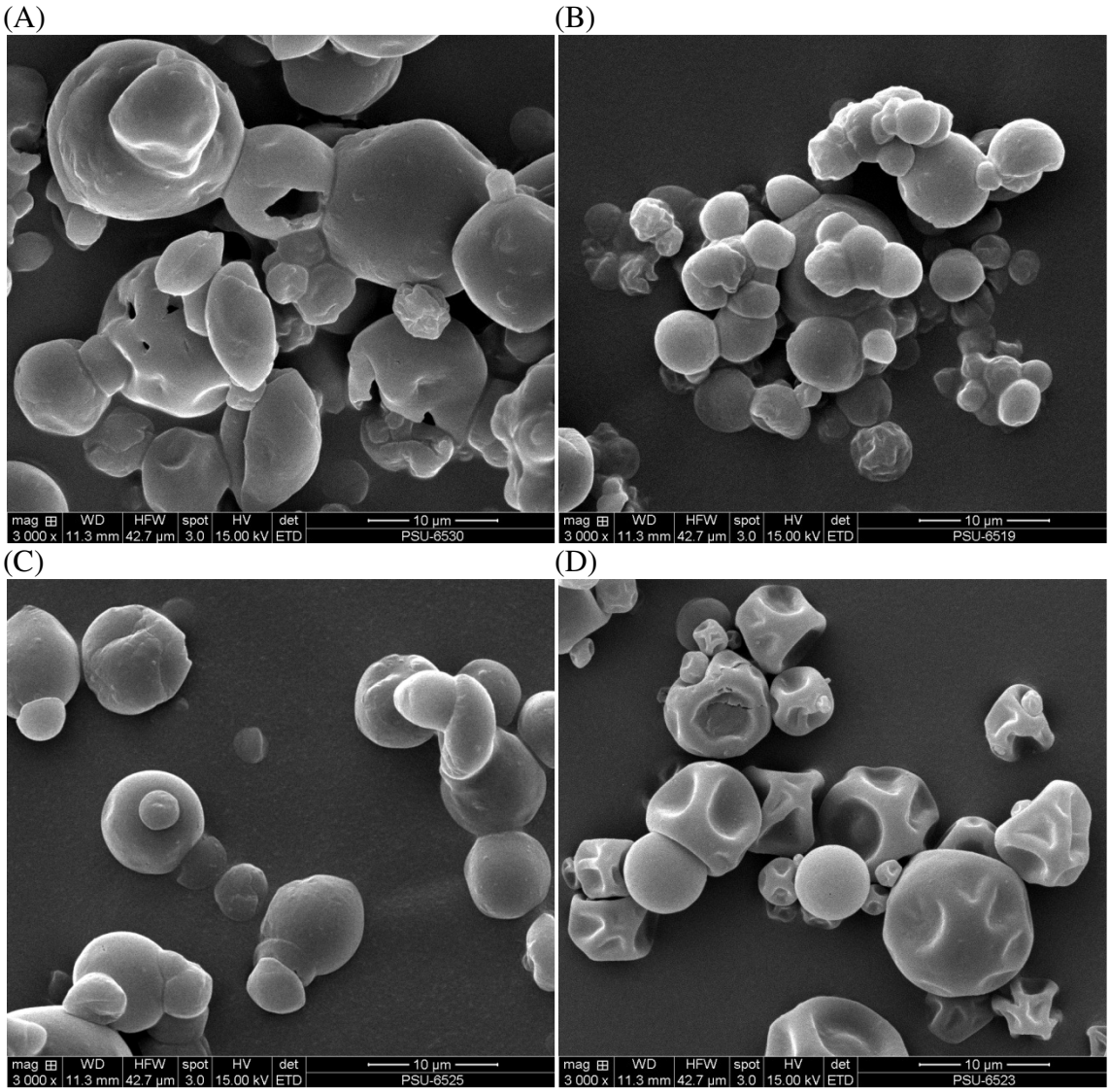


Figure 2 Scanning electron micrographs of fish oil microencapsule prepared using SRPH without and with TA or OTA as wall materials. Microcapsules prepared using SRPH (A); SRPH-TA (B); SRPH-OTA (C) and Ca-WPC (D). Magnification at 3,000x.

Nevertheless, the uniform wall with no leakage could be obtained for SRPH samples when TA or OTA was incorporated (Figure 26B, C). The agglomerated powders were found in all SRPH samples, particularly those containing TA. This might be attributed to the high moisture content and lower EE of SRPH sample. For hydrophilic wall material, a high moisture content could promote particle bridging/ agglomeration via vander Waals and electrostatic forces (Walton and Mumford, 1999). Furthermore, the

microspheres with excessive amounts of surface oil promoted significant agglomeration with reduced microencapsulation efficiency (Tan *et al.*, 2009). It was noted that the shrunk surface could be found in the microcapsule prepared using the mixture of Na-caseinate and whey protein concentrate as wall material (Figure 26D). The shrunk microcapsule of Ca-WPC sample might be caused by high viscosity of its infeed. Upon spray drying, the slower water diffusion and dehydration, compared to SRPH samples, could allow longer times to deform, shrink and collapse (Alamilla-Beltrán *et al.*, 2005). Spherical microcapsule with no fissure could be prepared by encapsulation of oil with casein hydrolysate (Molina Ortiz *et al.*, 2009). This led to the high fish oil retention in the matrix. Apart from moisture content, the morphology of the microcapsule was also related with drying temperature (Aghbashlo *et al.*, 2013b). When high temperature was used during drying, the dry crust of wall material was rapidly formed in the early stage of drying. The subsequent steam formation in the internal portion of the particle led to the formation of inflation of the particle called 'ballooning phenomenon' (Drusch *et al.*, 2007). The result suggested that wall material played a profound role in morphology of fish oil microcapsules. SRPH combination with OTA was found to render fish oil microcapsules with spherical shape and smooth surface.

8.4.2 Oxidative stability of fish oil microcapsules

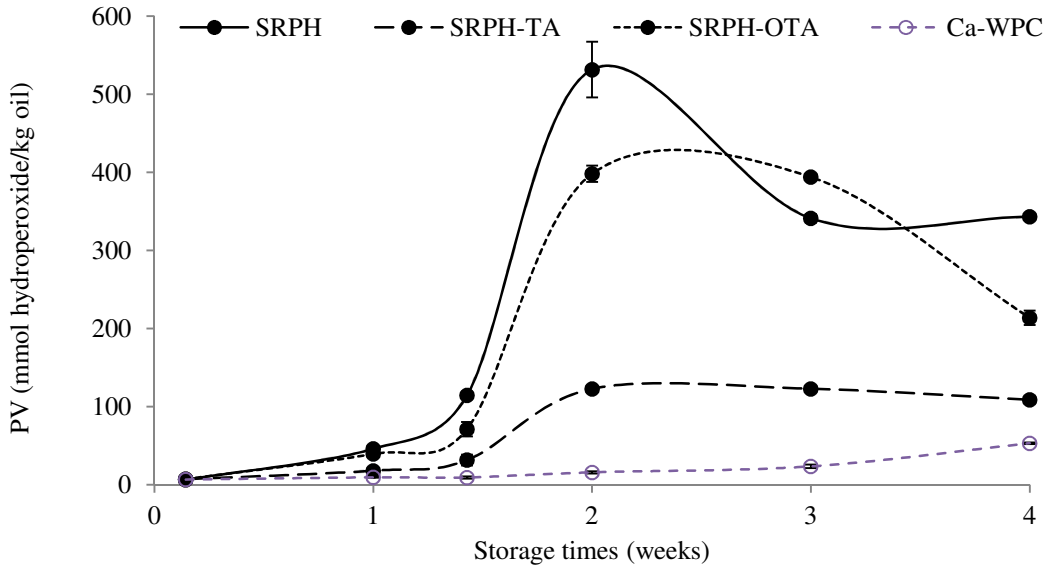
Oxidative stability of different fish oil microcapsules as monitored by PV and TBARS during 4 weeks of storage at 30 °C is shown in Figure 27. Slight increase in PV was found in all samples within the first week ($p < 0.05$). After week 1, the drastic increase in PV was observed in SRPH sample ($p < 0.05$). However, the lower PV was observed in SRPH when OTA was added ($p < 0.05$). TA incorporation showed the higher ability in prevention of PV formation, compared with OTA. When TA underwent oxidation, in which OTA was formed, antioxidative activity was decreased. This was evidenced by the higher PV of SRPH-OTA microcapsule. SRPH-TA sample had higher PV than Ca-WPC sample. The lower formation of hydroperoxide in Ca-WPC might be responsible to antioxidative properties of caseinate (Serfert *et al.*, 2009). Additionally, the higher EE of Ca-WPC indicated higher amount of lipids retained in the matrix, providing the effective protection

against oxidative deterioration caused by environmental exposure (light, oxygen, heat, etc.)

During 4 weeks of storage, PV increased in all samples ($p < 0.05$), up to 2 weeks. Thereafter, PV decreased until the end of storage (4 weeks). This might be attributed to the decomposition of hydroperoxides during the extended storage. Hydroperoxides were presumably degraded into secondary oxidation products such as aldehydes, ketones, alcohols, etc. (Anwar and Kunz, 2011; Chaiyasit *et al.*, 2007). Nevertheless, PV of Ca-WPC samples gradually increased throughout the storage of 4 weeks. The higher oxidative stability of Ca-WPC sample was in agreement with the high EE (Figure 25), in which higher amount of oil was trapped inside the wall. Furthermore, CA-WPC sample without pores (Figure 26D) might also reduce the oxygen penetration to the core matrix, leading to higher oxidative stability. Although SRPH-OTA sample showed the higher EE (Figure 25), this sample had the lower storage stability than SRPH-TA samples. This was more-likely due to the higher antioxidative activity of TA, a reduced form. Serfert *et al.* (2009) reported that the composite antioxidants including tocopherol, ascorbyl palmitate, lecithin and rosemary extract could retard auto-oxidation of fish oil microcapsule effectively.

Within the first 2 weeks of storage, TBARS of all samples dramatically increased ($p < 0.05$), particularly SRPH sample (Figure 27b). After 3 weeks, TBARS value of SRPH and SRPH-OTA samples slightly decreased. The addition of TA or OTA generally resulted in the lower increasing rate of TBARS ($p < 0.05$). The lower TBARS for microcapsule having SRPH and TA as wall materials was in agreement with lower PV, compared with those found in microcapsule using SRPH and OTA. Kolanowski *et al.* (2005) found that the addition of antioxidants (α-tocopherol, lycopene) at high level resulted in the higher PV, compared to those without antioxidants. The addition of those natural antioxidants at certain concentration could not increase oxidative stability, but they acted as prooxidants, particularly in the absence of chelating agents and reducing compounds (Kolanowski *et al.*, 2005). Thus, the addition of TA or OTA significantly retarded the autoxidation of fish oil microcapsule during storage.

(A)



(B)

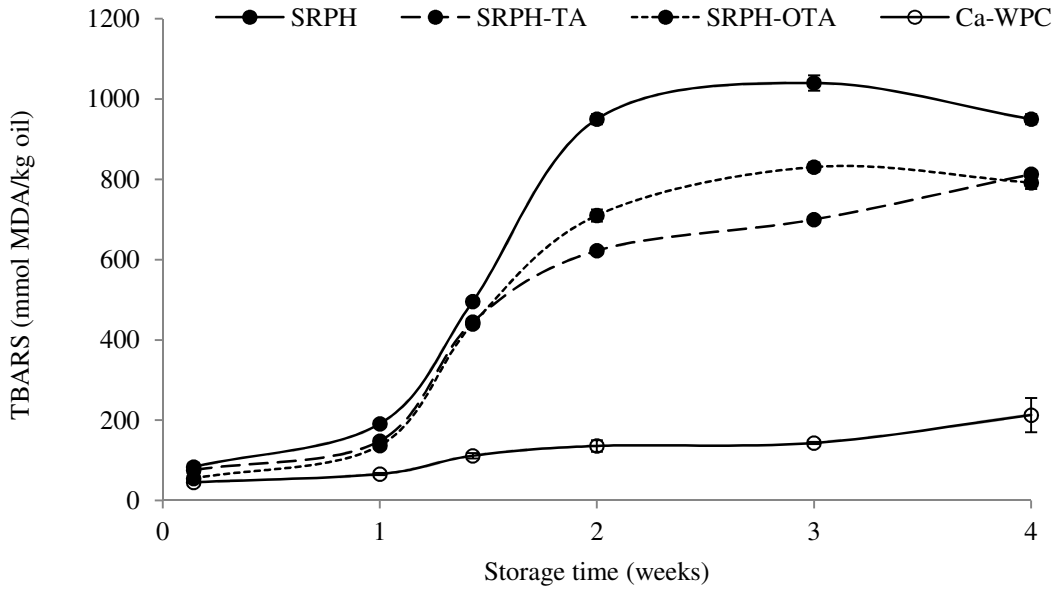


Figure 3 Changed in PV (A) and TBARS (B) of fish oil microcapsules prepared using SRPH without and with TA or OTA as wall materials during storage at 30 °C. Bars represent the standard deviation (n=3).

8.5 Conclusion

SRPH having 5% DH exhibited the poorer coating material for microencapsulation of fish oil using spray dryer, compared with Ca-WPC. Addition of TA or OTA decreased fish oil powder size with increasing EE. The addition of TA could increase the oxidative stability of fish oil microcapsule. Thus, other proteins could be further mixed with SRPH for increasing EE, while TA or OTA could be used as antioxidative cross-linker.

CHAPTER 9
CONCLUSION AND SUGGESTION

9.1 Conclusion

1. Roes from three species of tuna including skipjack, tongol and bonito were the promising sources of protein, fatty acid as well as minerals. Lipids were rich in docosahexaenoic acid (DHA) with the low cholesterol content. After defatting, the residue had high contents of protein and essential amino acids. Tuna roes can be therefore used as proteinaceous raw materials for preparation of food ingredient or other uses.

2. Skipjack roe protein hydrolysate (SRPH) prepared by Alcalase had antioxidant activity both radical scavenging activity and metal chelating activity. The obtained hydrolysates had high solubility and interfacial properties. SRPH with 5% DH, exhibiting the highest antioxidative and functional properties, contained two major peptides with MW of 57.8 and 5.5 kDa. Thus SRPH powder from skipjack could be alternatively served as an ingredient, which can serve as the emulsifier in food emulsion and simultaneously acts as an antioxidant to prevent the lipid oxidation. Thus, the safe nutritive additive can be used in food industry.

3. SRPH with 5% DH contained peptides possessing ACE inhibitory activity along with antioxidative activity. MLVFAV exhibited the highest inhibitory activity toward ACE, whilst DLDLRKDLYAN showed the highest metal chelating activity, ABTS radical and singlet oxygen scavenging activities. Therefore, SRPH could be potentially served as a functional food ingredient with pharmaceutical function against hypertension symptom and diseases associated with radicals.

4. SRPH with 5% DH could be used as the natural antioxidant to retard lipid oxidation in Broadhead catfish emulsion sausage enriched with polyunsaturated fatty acids (PUFAs) during the refrigerated storage. Thus, PUFA was more retained in the sausage added with SRPH. Addition of SRPH could improve textural properties but had no detrimental effect on the organoleptic properties. Therefore, SRPH having

5% DH, especially at a level of 3%, can be applied to improve the hardness and extend the shelf-life of fish sausage

5. Stability of emulsion was governed by homogenization pressure. Higher homogenization pressure reduced droplet size but decreased amount of adsorbed proteins. Emulsification at 2000 psi could provide the highest stability of SRPH containing emulsion during 14 days of storage. Addition of OTA with post-emulsification into emulsion containing SRPH as emulsifier could increase emulsion stability. The stability of emulsion was also affected by OTA concentration. The addition of OTA at least 1% was able to enhance the oxidative stability of menhaden oil-in-water emulsion

6. SRPH having 5% DH acted as the poor coating material for microencapsulation of fish oil using spray dryer, compared with Ca-WPC. Addition of TA or OTA decreased fish oil powder size with increasing EE. The addition of TA could increase the oxidative stability of fish oil microcapsule.

9.2 Suggestion

1. Protein hydrolysate with various degree of hydrolysis (DH) from other tuna should be prepared and evaluated for antioxidative activities and functional properties.

2. Mode of action of the ACE-inhibitory peptides from isolated SRPH with 5% DH should be characterized. The resistance of GI digestion stimulation on bioavailability of ACE-inhibitory peptide should be performed

3. Stability of emulsion containing SRPH (with 5% DH) and oxidized tannic acid (OTA) at different pHs (3-7) should be evaluated.

4. Microencapsulation using SRPH in combination with other proteins should be further studied to increase EE.

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