Proteases from Hepatopancreas of Pacific White Shrimp: Characteristics, Recovery and Applications

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ชื่อวิทยานิพนธ์ โปรตีเอสจากตับอ่อนกุ้งขาว: คุณลักษณะ การเก็บเกี่ยว และการประยุกต์ใช้

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บทคัดย่อ

ตับอ่อนเป็นอวัยวะที่อุดมไปด้วยโปรตีเอสโดยเฉพาะทริปซิน ทริปซินจากตับอ่อนของกุ้งขาวที่ผ่านการทำให้บริสุทธิ์โดยการตกตะกอนด้วยแอมโมเนียมซัลเฟต และด้วยชุดโครมาโทกราฟีซึ่งประกอบด้วยคอลัมน์ DEAE Sepharose และ SBTI-Sepharose 4B มีน้ำหนักโมเลกุลเท่ากับ 24 กิโลดัลตัน มีกิจกรรมสูงสุดเมื่อใช้ BAPNA เป็นสับสเตรทที่พีเอช 8.0 และอุณหภูมิ 60 °ซ และมีความคงตัวต่อความร้อนที่อุณหภูมิสูงถึง 60 °ซ และพีเอชช่วง 7.0-11.0 ทริปซินมีค่า k_{cat} สูงสำหรับ BAPNA

จากการศึกษาใช้ระบบการแยกชนิดสามเฟส (Three-phase partition system, TTP) ประกอบด้วยสารสกัดหยาบโปรตีเอส (Crude protease extract, CPE) และ *t*-บิวทานอลด้วยอัตราส่วน 1: 1 ในสภาวะที่มีแอมโมเนียมซัลเฟตร้อยละ 30 พบว่า สามารถแยกส่วนโปรตีเอสให้อยู่ระหว่างเฟส และเมื่อทำการแยกโปรตีเอสต่อด้วยระบบของเหลวสองเฟส (Aqueous two phase system, ATPS) ซึ่งประกอบด้วย PEG1000 (ร้อยละ 15, น.น./น.น.) และแมกนีเซียมซัลเฟต (ร้อยละ 25, น.น./น.น.) แล้วผ่านการทำบริสุทธิ์ต่อไปโดยการสกัดย้อนกลับ (Black extraction, BE) ที่ประกอบด้วย PEG8000 ร้อยละ 25 และแมกนีเซียมซัลเฟตร้อยละ 10 (น.น./น.น.) ได้ผลผลิตร้อยละ 46.16 โดยเอนไซม์ที่ได้มีความบริสุทธิ์เพิ่มขึ้น 9.94 เท่า ดังนั้นการใช้ระบบต่างๆร่วมกันในการแยก (TPP-ATPS-BE) สามารถเก็บเกี่ยว และทำบริสุทธิ์โปรตีเอสจากตับอ่อนกุ้งขาวอย่างมีประสิทธิภาพ

การศึกษาการย่อยสลายตัวเองที่อุณหภูมิ 60 °ซ พบว่า ไขมันถูกปลดปล่อยออกมาจากตับอ่อนกุ้งขาวเพิ่มขึ้น โดยมีผลผลิตไขมันเพิ่มขึ้นร้อยละ 7.4-8.8 เมื่อเวลาย่อยสลายตัวเองเพิ่มขึ้นจาก 0 เป็น 150 นาที นอกจากนี้ปริมาณแคโรทีนอยด์ทั้งหมดเพิ่มขึ้นเมื่อเวลาย่อยสลายตัวเองนานกว่า 60 นาที (p<0.05) อย่างไรก็ตามการเกิดออกซิเดชันของไขมันก็เพิ่มขึ้นเมื่อเวลาย่อยสลายตัวเองเพิ่มขึ้น(p<0.05) ไขมันที่สกัดได้มีกรด docosahexaenoic (DHA; C22: 6 (n-3)) สูงสุด และมีกรด eicosapentaenoic (EPA; C20: 5 (n-3)) รองลงมา ดังนั้นการย่อยสลายตัวเองที่อุณหภูมิ 60 °ซ เป็นเวลา 60 นาที สามารถเพิ่มผลผลิตของไขมันโดยไม่มีผลกระทบต่อคุณภาพ

เพื่อขยายการใช้ประโยชน์โปรตีเอสในตับอ่อนกุ้งขาวจึงทำการเตรียมสารสกัดหยาบโปรตีเอส (CPE) เพื่อใช้สำหรับผลิตโปรตีนไฮโดรไลเสต และแคโรทีโนโปรตีนพบว่าโปรตีนไฮโดรไลเสตจากหนังปลากะพงขาวซึ่งเตรียมโดยใช้ CPE หรือ อัลคาเลส มีกิจกรรมต้านอนุมูลอิสระ DPPH, ABTS และ FRAP เพิ่มขึ้นเมื่อระดับการไฮโดรไลซิสเพิ่มขึ้น (p<0.05) โปรตีนไฮโดรไลเสต (500-2000 มิลลิกรัม/ลิตร) สามารถยับยั้งการเกิดออกซิเดชันของไขมันในระบบเลซิทิน-ไลโพโซมได้เพิ่มขึ้นเมื่อปริมาณเพิ่มขึ้น จากการศึกษาน้ำหนักโมเลกุลของเปปไทด์ที่มีกิจกรรมต้านออกซิเดชัน ด้วยเทคนิคเจลฟิวเตรชันโครมาโทกราฟี โดยใช้คอลัมน์ Sephadex G-15 พบว่าเปปไทด์มีน้ำหนักโมเลกุล 364 ดัลตัน และแสดงกิจกรรมการกำจัดอนุมูลอิสระ ABTS ได้สูงสุด เมื่อสกัดแคโรทีโนโปรตีนจากเปลือกของกุ้งขาวโดยใช้ CPE พบว่า สามารถเก็บเกี่ยวแคโรทีโนโปรตีนเพิ่มขึ้นเมื่อระดับของโปรตีเอส และเวลาการย่อยสลายเพิ่มขึ้น แคโรทีโนโปรตีนมีแอสตาแซนทีน และแอสตาแซนทีนไดเอสเทอร์เป็นแคโรทีนอยด์หลัก แคโรทีโนโปรตีนมีฤทธิ์ต้านอนุมูลอิสระเพิ่มขึ้น ตามความเข้มข้นที่เพิ่มขึ้น แคโรทีโนโปรตีนประกอบด้วยปริมาณโปรตีนร้อยละ 73.58 ไขมันร้อยละ 21.87 และ เถ้าร้อยละ 2.63 และมีกรดแอสปาร์ติก/แอสพาราจีน และ กรดกลูตามิก/กลูตามีนเป็นกรดอะมิโนหลัก

เมื่อศึกษาการสกัดไขมันและน้ำมันจากเนื้อปลาสวาย โดยใช้ CPE ที่ระดับแตกต่างกัน (5-15 ยูนิต/กรัมโปรตีน) พบว่า ผลผลิตของไขมันเพิ่มขึ้นใน 30 นาทีแรกของการย่อยสลาย และการสกัดโดยใช้ CPE ร่วมกับแคลเซียมคลอไรด์เข้มข้น 50 มิลลิโมลาร์ ร่วมกับ CPE ให้ประสิทธิภาพในการสกัดใกล้เคียงกับอัลคาเลส อย่างไรก็ตามผลผลิตที่ได้ต่ำกว่าเมื่อเปรียบเทียบกับการสกัดด้วยตัวทำละลาย ไขมันที่สกัดได้ทั้งหมดมีไตรกลีเซอไรด์เป็นองค์ประกอบหลักและอุดมไปด้วยกรดปาล์มิติกและกรดโอเลอิก นอกจากนั้นเมื่อนำ CPE (5-15 ยูนิต/กรัมโปรตีน) ไปใช้ในการแยกน้ำมันมะพร้าวบริสุทธิ์จากน้ำกะทิที่อุณหภูมิห้อง (28-30 °ซ) พบว่าผลผลิตเพิ่มขึ้นใน 6 ชั่วโมงแรกของการแยก การใช้ CPE มีประสิทธิภาพใกล้เคียงกับกับกระบวนการหมักด้วยยีสต์ อย่างไรก็ตามไขมันที่แยกด้วยกระบวนการหมักเกิดปฏิกิริยาออกซิเดชันของน้ำมันในระดับที่สูงกว่าการใช้ CPEส่งผลเพิ่มค่าดัชนีการเกิดครีม และเหนี่ยวนำให้เกิดการรวมตัวของหยดน้ำมันในน้ำกะทิ เมื่อทำการย่อยสลายที่อุณหภมิ 60 °ซ ซึ่งเป็นอุณหภูมิที่เหมาะสมของทริปซินพบว่า การแยกน้ำมันใช้เวลาสั้นลง กว่าที่อุณหภูมิห้อง 4 เท่า อย่างไรก็ตามการใช้อัลคาเลสให้ประสิทธิภาพในการแยกน้ำมันมะพร้าวบริสุทธิ์ที่สูงกว่า CPE เล็กน้อย

ดังนั้นโปรตีเอสจากตับอ่อนกุ้งขาวจึงเป็นทางเลือกสำหรับใช้เป็นตัวช่วยในกระบวนการแปรรูปที่ให้ประสิทธิภาพใกล้เคียงกับโปรตีเอสทางการค้า

Thesis Title Proteases from hepatopancreas of Pacific white shrimp: Characteristics, recovery and applications

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ABSTRACT

Hepatopancreas was rich in proteases, particularly trypsin. Trypsin from hepatopancreas of Pacific white shrimp (*Litopenaeus vannamei*) was purified to homogeneity using ammonium sulfate precipitation and a series of chromatographies including DEAE sepharose and SBTI-Sepharose 4B columns. It had a molecular weight of 24 kDa with the optimal pH and temperature for α-*N*-benzoyl-dl-arginine-*p*-nitroanilide (BAPNA) hydrolysis of 8.0 and 60 °C, respectively. Trypsin was stable to heat treatment up to 60 °C, and over a pH range of 7.0–11.0. Trypsin showed high k_{cat} toward BAPNA.

Three phase partitioning (TPP) system comprising crude protease extract (CPE) and *t*-butanol with a ratio of 1:1 in the presence of 30% ammonium sulfate was used to fractionate proteases into the interphase. Subsequently, aqueous two phase system (ATPS) containing PEG1000 (15%, w/w) and $MgSO_4$ (25%, w/w) was applied. ATPS fraction was further subjected to back extraction (BE). BE using 25% PEG8000 and 10% $MgSO_4$ gave the yield of 46.16% with a purification fold of 9.94. The combined partitioning systems, TPP-ATPS-BE, could be effectively used to recover and purify proteases from hepatopancreas of Pacific white shrimp.

With enhanced autolysis at 60 °C, more lipids were liberated from hepatopancrease. Yield of lipid increased from 7.4 to 8.8% as autolysis time increased from 0 to 150 min. Coincidental increase in total carotenoid content was obtained with increasing autolysis time ($p<0.05$). Nevertheless, lipid oxidation increased when autolysis time increased ($p<0.05$). Lipids extracted contained docosahexaenoic acid (DHA; C22:6(n - 3)) as the most abundant fatty acid, followed by eicosapentaenoic acid (EPA; C20:5(n - 3)). Therefore, prior autolysis at 60 ^{o}C for 60 min was able to increase the yield without negative effect on lipid quality.

To widen the applications of proteases from hepatopancreas, CPE was prepared and further used for production of protein hydrolysate and carotenoprotein.

The hydrolysate from skin of seabass prepared using CPE or Alcalase had the increases in DPPH, ABTS radical scavenging activities and FRAP as DH increased (p<0.05). The hydrolysate (500-2000 mg/L) could inhibit lipid oxidation in a lecithin liposome system in a dose dependent manner. Based on gel filtration using a Sephadex G^{TM}-15 column, peptide with a MW of 364 Da showed the strongest ABTS radical scavenging activity. The recovery of carotenoprotein from shells of Pacific white shrimp with the aid of CPE was increased with increasing protease levels and hydrolysis times. Carotenoprotein contained astaxanthin and astaxanthin diester as major carotenoids. It showed increasing antioxidative activity when the concentrations increased. Carotenoprotein consisted of 73.58% protein, 21.87% lipids and 2.63% ash contents. Asp/Asn and Glu/Gln were found as the dominant amino acids in carotenoprotein.

CPE was also used for extraction of lipids and oils. Lipids from striped catfish muscle were extracted with the aid of CPE at different levels (5-15 unit/g protein) and the yield of lipids increased within the first 30 min of hydrolysis. Yield of lipid increased when 50 mM $CaCl_2$ was combined with CPE and exhibited similar extraction efficiency to Alcalase. However, the yield was lower than that obtained from solvent extraction. All lipid samples had triglycerides as the major component and were rich in palmitic acid and oleic acid. When virgin coconut oil (VCO) was separated from coconut milk with the aid of CPE at different levels (5-15 unit/g protein) at room temperature (28-30 °C), the yield increased within the first 6 h**.** The use of CPE exhibited similar separation efficiency to the typical fermentation process. However, lipids separated with fermentation process underwent oxidation to a higher extent, compared with those separated by CPE. CPE increased the creaming index and induced the collapse of oil droplets in coconut milk. When the hydrolysis was performed at 60 °C, an optimal temperature for trypsin, the shorter time (90 min) was required for VCO separation. However, Alcalase showed higher efficacy in VCO separation, compared with CPE and the control (without enzyme) (p<0.05). VCOs were rich in lauric acid (C12:0) and myristic acid (C14:0).

Therefore, proteases from hepatopancrease of Pacific white shrimp could be used as an alternative processing aid in which their efficiency was similar to commercial protease.

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CHAPTER 1
INTRODUCTION AND REVIEW OF LITERATURE

1.1 Introduction

Pacific white shrimp (*Litopenaeus vannamei*) accounts for 90% of the global aquaculture shrimp production (Wyban, 2007). Thailand has been a major shrimp exporter for over 15 years (Thai Shrimp Association, 2013) and is one of current leaders in shrimp production, providing more than 500,000 tons in 2012, which accounts for 15 % of the world production (Anderson and Valderrama, 2013). Thailand's shrimp production is valued at approximately US $3 billion in 2012, and the current major shrimp markets include the USA (38 %), Japan (23 %) and the European Union (15 %) (Sombatjinda *et al.*, 2014). The crustacean processing industry generates a large amount of by-products (mainly exoskeleton and cephalothorax), which can represent around 50-70% of the total weight of raw material. Those by-products are the rich sources of biomolecules including lipid, carotenoid, chitin, proteins, flavorants, nutritive components and enzymes (Sila *et al.*, 2012a). By-products are generally considered as low market value matter and mainly used as feeds or fertilizer. Additionally, the disposal and treatment of crustacean processing byproducts frequently require additional costs (Arancibia *et al.*, 2014). With improper disposal, they can cause the pollution. To exploit those left-over, cephalothoraxes have been used as raw material for production of shrimp hydrolysate, shrimp flavorant, carotenoid and chitin/chitosan (Benhabiles *et al.*, 2012; Khan and Nowsad, 2013; Robert *et al.*, 2014; Sowmya and Sachindra, 2012).

Hepatopancreas free-whole shrimp is another product with increasing demand. During processing, hepatopancreas is removed by a vacuum sucking machine and can serve as the essential source of protease, proteins, lipids and carotenoids (Takeungwongtrakul *et al.*, 2012). Lipids from fish and crustacean have been paid increasing interest as the important source of n-3 fatty acids, mainly DHA and EPA, which have been known for health benefit (Mahaffey, 2004). In addition, astaxanthin has been reported as the main carotenoid found in shrimp by-product (Sánchez-Camargo *et al.*, 2011). Astaxanthin was reported to be 10-fold stronger

antioxidant activity than that of other carotenoids, namely zeaxanthin, lutein, canthaxanthin and carotene and 100-fold greater than that of α-tocopherol (Takeungwongtrakul *et al.*, 2014). Carotenoids were extracted using traditional solvent extraction, supercritical fluid extraction (Babu *et al.*, 2008) and vegetable oils (Sachindra and Mahendrakar, 2005). The use of proteases is another means to extract carotenoproteins from shrimp processing byproducts (Cano-Lopez *et al.*, 1987; Chakrabarti, 2002)

Virgin coconut oil (VCO) is growing in popularity as functional food and its public awareness is increasing. Coconut oil is extensively used for food and industrial purposes (Marina *et al.*, 2009a). VCO is rich in medium chain fatty acids (MCFA) and exhibits good digestibility (Norulaini *et al.*, 2009). VCO can be produced using several techniques as follows: (1) drying the freshly grated coconut meat at low temperature, no higher than 60°C, followed by pressing to extract the VCO; (2) by extracting the coconut milk from the freshly grated coconut meat, followed by the addition of enzyme or aging for several hours, or by mechanical process using continuous centrifuge. The enzyme-assisted coconut oil extraction significantly increased the oil yield in aqueous system (Tano-Debrah and Ohta, 1997). Enzymatic extraction is effective in releasing the oil by breaking down the coconut emulsion (Man *et al.*, 1996; Seow and Gwee, 1997). Protease from Pacific white shrimp hepatopancreas could serve as an aid for a wide range of applications. Although microbial proteases have been widely used for hydrolysis, the cost of enzyme is another concern for user or industry. Thus, the proteases from cheap sources, especially shrimp processing byproducts, e.g. viscera can be alterative and promising tool for protein cleavage. With appropriate hydrolysis, mediated by shrimp protease, the complex systems could be destroyed, in which the target substrate or products could be released or obtained. Therefore, the cost of commercial protease is lowered and shrimp processing by products is better exploited. The information gained could provide the better understanding and the alternative uses of protease from shrimp processing byproduct can be achieved.

1.2 Review of Literature

1.2.1 Fish and shellfish enzymes

1.2.1.1 Protease

Digestive organs are rich in proteases and play a role in digestion for fish and shellfish. Hepatopancreas can serve as the excellent source of proteases (Jeong *et al.*, 2000). However, other digestive glands, such as pyloric caeca, stomach, spleen, etc. have been reported to contain proteases (Ketnawa *et al.*, 2013; Klomklao *et al.*, 2004).

1.2.1.1.1 Classification of fish and shellfish proteases

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

- **Aspartyl protease**

Aspartyl or acid proteases belong to a family of proteolytic enzymes that use an aspartate residue for catalysis of their peptide substrates. In general, they have two highly-conserved aspartates in the active site. Aspartyl proteases are inhibited by pepstatin. Aspartyl proteases have been described as a group of endopeptidase characterized by high activity and stability at acidic pH (Tang and Wong, 1987). Their catalytic sites are composed of the carboxyl groups of two aspartic acid residues (Simpson, 2000). Based on the EC system, all aspartyl proteases from marine animals have the first three digits in common: EC 3.4.23. Three types of aspartyl proteases that have been isolated and characterized from the stomachs of marine animals are pepsin, chymosin and gastricsin (Simpson, 2000).

Pepsin is composed of a single polypeptide chain of 321 amino acids and has a molecular weight (MW) of 35 kDa (Simpson, 2000). Pepsin, secreted as a zymogen (pepsinogen), is activated by the acid in stomach to an active form (Clark *et al.*, 1985). Pepsin prefers specifically the aromatic amino acids, involving phenylalanine, tyrosine and tryptophan. Fish pepsins have been isolated from various fish species such as Atlantic cod (Gildberg and Øverbø, 1990). However, the pepsins

from marine animals were reported to have MW ranging from 27 to 42 kDa. MW of two pepsins (I and II) from pectoral rattail (*Coryphaenoides pectoralis*) stomach were estimated to be approximately 35 and 31 kDa, respectively, as estimated by SDS-PAGE and gel filtration on Sephacryl S-200 (Klomklao *et al.*, 2007c). Sánchez-Chiang *et al.* (1987) reported that MWs of two pepsins from stomach of salmon were estimated to be approximately 32 and 27 kDa by gel filtration. MWs of two pepsins from polar cod stomach were estimated by SDS-PAGE to be approximately 42 and 40 kDa (Arunchalam and Haard, 1985). Pepsins and pepsin-like enzymes can be extracted from the digestive glands of marine animals such as Atlantic cod (*Gadus morhua*) (Brewer *et al.*, 1984), capelin (*Mallotus villosus*) (Gildberg, 1983), polar cod (*Boreogadus saida*) (Arunchalam and Haard, 1985), sardine (*Sardinos melanostica*) (Noda and Murakami, 1981), Montery sardine (*Sardinops sagax caerulea*) (Castillo-Yanez *et al.*, 2004), Antarctic rock cod (*Trematomus bernacchii*) (Brier *et al.*, 2007), snakehead (*Channa argus*) (Chen *et al.*, 2009), albacore tuna (*Thunnus alalunga*) (Nalinanon *et al.*, 2010) and largemouth bass (*Micropterus salmoides*) (Miura *et al.*, 2015)

- Serine proteases

The serine proteases are a group of endopeptidase with a serine residue together with an imidazole group and an aspartyl carboxyl group in their catalytic sites (Simpson, 2000). The proteases in serine subclass have the same first three digits on the EC system as EC 3.4.21. Serine proteases exhibit high activity under alkaline rather than neutral pH have high sensitivity to serine protease inhibitors (Simpson, 2000). In mammals, serine proteases perform many important functions, especially in digestion, blood clotting, and the complement system (Simpson, 2000). The common serine proteases from hepatopancreas of shirmp are trypsin (EC 3.4.21.4), chymotrypsin (EC 3.4.21.1) and elastase (EC 3.4.21.11) (Aoki *et al.*, 2004). Trypsin cleaves peptide chains mainly at the carboxyl side of the amino acids lysine or arginine, except when either is followed by proline, while chymotrypsin preferentially cleaves peptide amide bonds where the carboxyl side of the amide bond is tyrosine, tryptophan, or phenylalanine. These amino acids contain an aromatic ring in their side chain that fits into a hydrophobic domain of the enzyme. Elastase exhibits preferential

specificity for alanine, valine and glycine (Simpson, 2000). Trypsin has been isolated and characterized from the hepatopancreas of various shrimp species, including white shrimp (*Paneaus setiferus*) (Gates and Travis, 1969), Indian prawn (*Penaeus indicus*) (Honjo *et al.*, 1990), Chinese white shrimp (*Penaevs omentaim*) (Oh *et al.*, 2000), Crayfish (*Pocambarus clarkii*) (Kim *et al.*, 1994), Northern shrimp (*Pandalus eous*) (Aoki *et al.*, 2003), North Pacific krill (*Euphausia pacifica*) (Wu *et al.*, 2008), freshwater prawn (*Macrobrachium rosenbergii*) (Sriket *et al.*, 2012), Antarctic krill (*Euphausia superba*) (Wu *et al.*, 2014), lobster (*Panulirus argus*) (Perera *et al.*, 2015) and royal red prawn (*Haliporoides sibogae*) (Sriket and Sriket, 2015).

- **Cysteine proteases**

Thiol or cysteine proteases are a group of endopeptidases that have cysteine and histidine residues as the essential groups in their catalytic sites. These enzymes require the thiol (-SH) group furnished by the active site cysteine residue to be intact, hence this group is named “thiol” or “cysteine” proteases (Mihalyi, 1978). The thiol proteases are inhibited by heavy metal ions and their derivatives, as well as by alkylating agents and oxidizing agents (Mihalyi, 1978). The first three digits common to thiol proteinases are EC 3.4.22.

Digestive cysteine or thiol proteases have been found in digestive organ of marine animals. Marine digestive cysteine proteases are most active at acidic pH and inactive at alkaline pH. Common examples of digestive thiol protease from marine animals are cathepsin B, cathepsin L and cathepsin S (Simpson, 2000). Various researchers have described different procedures for isolating marine cysteine or thiol proteinases from the digestive glands of marine animals. Cathepsin B was isolated from hepatopancreas of carp (Aranishi *et al.*, 1997). Cathepsin L was obtained from hepatopancreas of jumbo squid (Cardenas-Lopez and Haard, 2009).

- **Metalloproteases**

The metalloproteases have a common first three digits as EC 3.4.24 (Simpson, 2000). The metalloproteases are hydrolytic enzymes whose activity depends on the presence of bound divalent cations (Simpson, 2000). Chemical

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

1.2.1.1.2 Fish trypsins/chymotrypsins

Trypsin is one of the main proteases in digestive tract of fish and crustaceans. Trypsin has a catalytic triad of three essential amino acid residues including serine, histidine and aspartate within the S1 binding pocket. The high specificity of trypsin for lysine and arginine results from the negative charge of aspartate at the S1 binding pocket of trypsin, which can match the positive charge of the P1 side chain of the substrate (Kishimura *et al.*, 2007). In the shrimp hepatopancreas, trypsin not only functions as a digestive enzyme, but also is responsible for activating all the pancreatic enzymes by cleaving a short activation peptide from the amino-terminus of inactive zymogens (Gates and Travis, 1969).

Crustacean trypsin has low thermal stability but shows high activity at low temperature. Furthermore, it is unstable at acidic pH, and contains a lower content of basic amino acid residues in the polypeptide chain in comparison with mammalian trypsins (Gates and Travis, 1969). Trypsin consists of single peptide chain with MW of 24 kDa (Klomklao *et al.*, 2007c). MW of fish trypsins can vary with species. The optimal pH for the activation is between pH 7.5 and 10.5. This enzyme is more selective than any other enzymes, cleaving peptide bonds at the carboxyl terminus of lysine and arginine residues exclusively (Klomklao *et al.*, 2007c).

Trypsins have been isolated and characterized from hepatopancreas, or intestines of several animals. Sriket *et al.* (2011) reported that the highest proteolytic activity of crude protease extract from the hepatopancreas of freshwater prawn (*Macrobrachium rosenbergii*) was found at pH 7 and 60 °C when casein was used as a substrate. Major proteases isolated from hepatopancreas of Northern shrimps (*Pandalus borealis*) were classified as serine proteases (Aoki *et al.*, 2003). Extract of *Penaeus vannamei* hepatopancreas consisted of trypsin and chymotrypsin (Ezquerra *et al.*, 1997). Proteolytic activities were found in hepatopancreas of the crustaceans Munida. Trypsin-like activity was higher than chymotrypsin-like activity (Rossano *et al.*, 2011). Sriket *et al.* (2012) purified trypsin from hepatopancreas of freshwater prawn (*Macrobrachium rosenbergii*) using a series of chromatographies including Q-Sepharose, Superdex 75 and MonoQ columns. The purified trypsin had a MW of 17 kDa. The optimal pH and temperature for Boc-Val-Pro-Arg-MCA hydrolysis were 8.0 and 55 °C, respectively. Trypsin was stable to heat treatment up to 40 °C, and over a pH range of 7.0–11.0. Sriket and Sriket (2015) also purified trypsin from hepatopancreas of royal red prawn by ammonium sulphate (40–60% saturation) precipitation, Benzamidine affinity column and MonoQ column chromatography. Trypsin showed a single band on native-PAGE. Trypsin had a MW of 27 kDa. The optimal pH and temperature for Boc-Val-Pro-Arg-MCA hydrolysis were 9.0 and 50°C, respectively. Purified enzyme was stable to heat treatment up to 50°C and over a pH range of 7.0-11.0. Five trypsin-like enzymes were purified from lobster (*Panulirus argus*) by a combination of size exclusion (Sephadex G-75 column) and anion exchange (MonoQ anion exchange column) chromatographies. The MWs of the enzymes ranged from 34.94 to 36.26. Optimal pH for most isoforms ranged from 7 to 8 and activity remained high in a broad alkaline range (Perera *et al.*, 2015). Wu *et al.* (2014) reported that three trypsins purified from Antarctic krill (*Euphausia superba*) by ammonium sulfate precipitation, ion-exchange and gel-filtration chromatography had relative MWs of 28.7, 28.8 and 29.2 kDa. Trysins had the optimum temperature at 40 (Trypsin I), 45 (Trypsin II) and 40 °C (Trypsin III) using BAPNA as a substrate. The trypsin was stable between 5 and 40 °C. Trypsin in hepatopancreas of shrimp *Penaus indicus* had MW of 36 kDa. The enzyme showed broad pH optimal (pH 6.5 - 11.0) and stability (pH 6.0-12.0), and was most active at 45 °C (Honjo *et al.*, 1990).

Wu *et al.* (2008) purified trypsin from North Pacific krill (*Euphausia pacifica*) by ammonium sulfate precipitation, ion-exchange and gel-filtration chromatography. MW of trypsin was 33 kDa. It was active over a wide pH (6.0–11.0) and temperature (10–70°C) range. It had the optimum pH of 9.0 and optimal temperature of 40–50°C. Trypsin was stable between pH 6.0 and 11.0 and below 30°C. Trypsin from Antarctic krill processing wastewater was purified using affinity chromatography on *p*-aminobenzamidine Sepharose 4B. It had MW between 32 and 33 kDa. The optimal pH and temperature were pH 8 and 60°C, respectively (Bustos *et al.*, 1999). (Dendinger and O'Connor, 1990) purified a 33.5 kDa trypsin from the midgut gland of Atlantic blue crab by a combination of molecular sieving, ion exchange, and hydrophobic chromatography and the maximal activity was found at 50°C and pH 8.2. Trypsin from hepatopancreas of the crab *Paralithodes camtschaticus* was isolated in homogeneous state by a successive ion-exchange chromatography on DEAE-Sephadex, affinity chromatography on Agarose modified with peptide ligands from trypsin hydrolysate of salmine, and ion-exchange chromatography on a Mono Q column. The trypsin contained 237 amino acids which correspond to its MW of 24.8 kDa (Kislitsyn *et al.*, 2003). Jeong *et al.* (2000) reported that trypsin A, B, C and D purified from the hepatopancreas of crawfish with MW of 35.0, 41.2, 37.9 and 39.5 kDa, respectively, were purified using DEAE Sepharose chromatography. They were inhibited by phenyl methyl sulfonyl fluoride (PMSF), and soybean trypsin inhibitor (SBTI). It exhibited the maximal activity at 60-70 °C and at pH 8.0-8.5. Trypsin from the hepatopancreas of *Sepia officinalis* was purified by fractionation with ammonium sulphate, Sephadex G-100 gel filtration, DEAE-cellulose ion-exchange chromatography, Sephadex G-75 gel filtration and Q-Sepharose anion-exchange chromatography. It had MW of 24 kDa and exhibited the maximal activity at 70°C, and pH 8 using BAPNA as a substrate (Balti *et al.*, 2009). Cao *et al.* (2000) reported that two anionic trypsins (trypsin A and trypsin B) from the hepatopancreas of carp were purified using a series of chromatographies including DEAE-Sephacel, UltrogelAcA54 and Q-Sepharose. Trypsin A with a MW of 28 kDa was purified to homogeneity, while trypsin B showed two bands of 28.5 kDa and 28 kDa on SDS-PAGE. Trypsin A and B had optimal activity at 40 and 45°C, respectively, and had the optimum pH of 9.0 using Boc-Phe-Ser-Arg-MCA as a substrate. Trypsin from

digestive gland of the shrimp *Penaeus japonicus* was also purified. It had the MW of 25 kDa with the optimal pH and temperature of 8-8.3 and 60°C, respectively (Galgani *et al.*, 1985). Additionally, trypsin from the hepatopancreas of the sand crab (*Portunus pelngicus*) was purified (Dionysius *et al.*, 1993). The MW of the trypsin estimated by gel filtration and mass spectrometry was 25 kDa and the optimal pH and temperature were 8 and 60°C, respectively. The optimal conditions of fish and shellfish trypsins are summarized in Table 1.

Table 1. Optimal conditions of various fish and shellfish trypsins

Identified species	**Optimum pH**	**Optimum temperature (ºC)**	**Substrates**	**References**
Shrimp (*Penaeus japonicus*)	8.0-8.3	60	BAPNA[a]	Galgani *et al.* (1985)
Shrimp (*Penaus indicus*)	6.5-11.0	45	BAPNA	Honjo *et al.* (1990)
Atlantic blue crab (*Callinectes sapidus*)	8.2	50	BAPNA	Dendinger and O'Connor (1990)
Antarctic krill (*Euphausia superba*)	8	60	BAPNA	Bustos *et al.* 1999)
Sand Crab (*Portunus pelagicus*)	8	60	TAME[b]	Dionysius *et al.* (1993)
Crawfish *(Procambarus clarkii)*	8.0-8.5	60-70	TAME	Jeong *et al.* (2000)
Starfish (*Asterina Pectinifera*)	8	55	TAME	Kishimura and Hayashi (2002)
King crab (*Paralithodes camtschaticus*)	-	-	BAPNA	Kislitsyn *et al.* (2003)
Monterey sardine (*Sardinops sagax caerulea*)	8	50	BAPNA	Castillo-Yanez *et al.* (2004)
White shrimp (*Penaeus vannamei*)	8-10	60	TAME	Sainz *et al.* (2004)
Japanese anchovy (*Engraulis japonica*)	8	60	TAME	Kishimura *et al.* (2005)
Bigeye snapper (*Pricanthus macracanthus*)	8-11	55	BAPNA	Van Hau and Benjakul (2006)
Chinook salmon (*Oncorhynchus tshawytscha*)	8	60	BAPNA	Kurtovic *et al.* (2006)
New Zealand hoki (*Macruronus ovaezealandlae*)	9	60	BAPNA	Shi *et al.* (2007)
Bluefish (*Pomatomus saltatrix*)	9.5	55	BAPNA	Klomklao *et al.* (2007b)

Table 1. Optimal conditions of various fish and shellfish trypsins (cont.)

Identified species	Optimum pH	Optimum temperature (°C)	Substrates	References
Skipjack tuna (*Katsuwonus pelamis*)	8.5	60	TAME	Klomklao *et al.* (2007a)
Spotted goatfish (*Pseudupeneus maculatus*)	9	55	BAPNA	Souza *et al.* (2007)
Jacopever (*Sebastes schlegelii*)	8	60	TAME	Kishimura *et al.* (2007)
Elkhorn sculpin (*Alcichthys alcicornis*)	8	50	TAME	Kishimura *et al.* (2007)
Walleye pollock (*Theragra chalcogramma*)	8	50	TAME	Kishimura *et al.* (2008)
North Pacific krill (*Euphausia pacifica*)	9	40-50	BAPNA	Wu *et al.* (2008)
Pacific cod (*Gadus macrocephalus*)	8	50	TAME	Fuchise *et al.* (2009)
Saffron cod (*Eleginus gracilis*)	8	50	TAME	Fuchise *et al.* (2009)
Hybrid tilapia (*Oreochromis niloticus* x *O. aureus*)	9	60	Casein	Wang *et al.* (2009)
Cuttlefish (*Sepia officinalis*)	8	70	BAPNA	Balti *et al.* (2009)
Smooth hound (*Mustelus mustelus*)	8.5	50	TAME	Bougatef *et al.* (2010a)
Threadfin hakeling (*Laemonema longipes*)	8	50	TAME	Kishimura *et al.* (2010)
Brownstripe red snapper (*Lutjanus vitta*)	8.5	60	BAPNA	Khantaphant and Benjakul (2010)
Barbel (*Barbuds callensis*)	10	55	BAPNA	Sila *et al.* (2012b)
Hybrid catfish (*Clarias macrocephalus* × *Clarias gariepinus*)	8	60	TAME	Klomklao *et al.* (2011)
Sardinelle (*Sardinella aurita*)	9	50-55	BAPNA	Khaled *et al.* (2011)
Silver mojarra (*Diapterus rhombeus*)	8.5	55	BAPNA	Silva *et al.* (2011)
Zebra blenny (*Salaria basilisca*)	9.5	60	BAPNA	Ktari *et al.* (2012b)
Freshwater prawn (*Macrobrachium rosenbergii*)	8.0	55	Boc-Val-Pro-Arg-MCA	Sriket *et al.* (2012)
Antarctic krill (*Euphausia superba*)	8.0	40-45	BAPNA	Wu *et al.* (2014)
Lobster (*Panulirus argus*)	7.0-8.0	60	BAPNA	Perera *et al.* (2015)
Royal red prawn (*Haliporoides sibogae*)	9.0	50	Boc-Val-Pro-Arg-MCA	Sriket and Sriket (2015)

[a] *α-N*-benzoyl-DL-arginine-*p*-nitroanilide
[b] *α-N-ρ*-toluene-sulfonyl-L-arginine methyl ester

Apart from trypsin, chymotrypsin is also found in fish and shellfish. Chymotrypsins, which cleave the peptides on the carboxyl side of phenylalanine, tyrosine and tryptophan residues, have been isolated and characterized from several fish including common carp (*Cyprinus carpio*) (Cohen *et al.*, 1981), dogfish (*Squalus acanthias*) (Racicot and Hultin, 1987), cod (*Gadus morhua L.*) (Raae and Walther, 1989), Atlantic cod (*G. morhua*) (Ásgeirsson and Bjarnason, 1993), anchovy (*Engraulis japonica*) (Heu *et al.*, 1995), grass carp (*Ctenopharyngodon idellus*) (Fong *et al.*, 1998) and Monterey sardine (*Sardinops sagax caeruleus*) (Castillo-Yánez *et al.*, 2006). Although chymotrypsins from fish are basically similar to mammalian counterparts, differences in structural and functional properties were reported (Fong *et al.*, 1998). Chymotrypsin A and B have been purified from the hepatopancreas of Crucian carp (*Carassius auratus*) using a series of chromatographies including DEAE-Sepharose, Sephacryl S-200 HR, Phenyl-Sepharose and SP-Sepharose. MW of chymotrypsin A and B were approximately 28 and 27 kDa, respectively. Optimum temperatures of chymotrypsin A and B were 40 and 50 °C, and optimal pHs were 7.5 and 8.0 using Suc-Leu-Leu-Val-Tyr-AMC as a substrate (Yang *et al.*, 2009). Hernández-Cortés *et al.* (1997) purified chymotrypsin from hepatopancreas of the white shrimp, *Penaeus vannamei*, by Q-Sepharose fast flow and and Phenyl- Q-sepharose column chromatography. MW of chymotrypsin was 33.2 kDa. It had the optimum pH of 8.0 when SAAPFNA was used a substrate. The enzyme is thermostable both at 25 and 37 °C. The MW of the purified chymotrypsin from the hepatopancreas of cuttlefish (*Sepia officinalis*) was estimated to be 28 kDa. The optimum pH and temperature for chymotrypsin were pH 8.5 and 55 °C, respectively, using SAAPFNA as a substrate. The enzyme was extremely stable in the pH range of 7.0-10.0 and highly stable up to 50 °C after 1 h incubation. The N-terminal amino acid sequence of the first 20 amino acids of the purified chymotrypsin was IVGGQEATIGEYPWQAALQV (Balti *et al.*, 2012). Chymotrypsin was purified from the midgut gland of yellow leg shrimp (*Penaeus californiensis*) using affinity chromatography (trypsin inhibitor-agarose gel (SBTI) columns), followed by preparative electrophoresis (Navarrete-del-Toro *et al.*, 2015). Chymotrypsin had a MW of 35.7 kDa. The optimal pH and temperature for SAAPFNA hydrolysis were

7.0 and 50 °C, respectively. Chymotrypsin was stable at 50°C and sensible to low pH (Navarrete-del-Toro *et al.*, 2015) *Pterygoplichthys disjunctivus* viscera chymotrypsin was purified by fractionation with ammonium sulfate (30–70 % saturation), gel filtration, affinity, and ion exchange chromatography (Villalba-Villalba *et al.*, 2013). Chymotrypsin had MW of approximately 29 kDa. The chymotrypsin exhibited maximal activity at pH 9 and 50 °C, using SAAPNA as substrate (Villalba-Villalba *et al.*, 2013).

1.2.1.2 Lipase and phospholipase

Lipase is an enzyme that catalyzes the formation or cleavage of lipids. Lipases belong to a subclass of the esterases (Xu, 2000). Most lipases act at a specific position on the glycerol backbone of lipid substrate to monoglycerides and two fatty acids (Arni and Ward, 1996). Several lipases exist in nature, such as phospholipases and sphingomyelinases. However these are usually treated separately from "conventional" lipases (Xu, 2000). A phospholipase is an enzyme that hydrolyzes phospholipids into fatty acids and other lipophilic substances. There are four major classes, termed A, B, C and D, distinguished by the type of reaction which they catalyze lipolysis (Xu, 2000).

The hepatopancreas is generally assumed to be the major source of digestive lipase in fish as it is in mammal (Tocher, 2003). However, digestive lipase may also be secreted by intestinal mucosa as several studies have found high lipase activity in mucous layers or intestinal segments of many fish species. These lipases may actually be of pancreatic origin, resulting from adsorption of pancreatic enzyme into intestinal mucosa (Tocher, 2003). However, in African catfish (*Clarius gariepinus*) and grass carp (*Ctenophearyngodon idella*), lipase activity was found in intestinal segment even after 48 and 24 h starvation, respectively (Das and Tripathi, 1991).

A lipase was purified from hepatopancreas of red sea bream by fractional precipitation with ammonium sulfate and sequential chromatographies. MW of lipase was 64 kDa. The purified lipase had a pH optimum in the pH range of 7.0–9.0 using *p*-nitrophenyl myristate or triolein as a substrate. The enzyme preferentially hydrolyzed ethyl esters of polyunsaturated fatty acid, such as

arachidonic acid and eicosapentaenoic acid, which were resistant to porcine pancreatic lipase (Iijima *et al.*, 1998). Takahashi *et al.* (1996) reported that lipase from hepatopancreas of the neon flying squid (*Ommastrephes bartramii*) had the MW of 33 kDa. Optimum pH and temperature were 7.0 and 25 °C. The enzyme was tolerably stable up to 37 °C. The enzyme appeared to have specific activity on monounsaturated and saturated fatty acids in triglyceride, most likely oleic and/ or palmitic acid. Two phospholipase A2 (PLA2) isoforms, tentatively denoted as DE-1 and DE-2 PLA2s, were purified from the hepatopancreas of red sea bream (*Pagrus major*) by sequential column chromatography on S-Sepharose fast flow, DEAE-Sepharose fast flow butyl-Cellulofine, and by ionexchange, gel-filtration and reversed-phase HPLC. The purified DE-1 and DE-2 PLA2s had MW of approx. 13.5 kDa. DE-1 PLA2 had a pH optimum at pH 10 using 2 mM phosphatidylcholine and phosphatidylethanolamine as substrates. DE-2 PLA2 also had a pH optimum in the alkaline region at around pH 8-9 using 2 mM of phosphatidylcholine as a substrate (Ono and Iijima, 1998). Lipase was purified from midgut gland of white leg shrimp (*Penaeus vannamei*). Pure lipase was obtained after Superdex 200 gel filtration and Resource Q anionic exchange. The pure lipase had a MW of about 44.8 kDa. The lipase hydrolysed short and long-chain triacylglycerols and naphthol derivates at comparable rates. Specific activities of 1787 Umg^{-1} and 475 Umg^{-1} was obtained when triolein and tributyrin were used as substrates, respectively,when tested at pH 8.0 and 30°C (Rivera-Pérez *et al.*, 2011).

1.2.1.3 Phosphatase

A phosphatase is an enzyme that removes a phosphate group from its substrate by hydrolyzing phosphoric acid monoesters into a phosphate ion and a molecule with a free hydroxyl group (Raimi *et al.*, 2012). This action is directly opposite to that of phosphorylases and kinases, which attach phosphate groups to their substrates by using energetic molecules like ATP. A common phosphatase in many organisms is alkaline phosphatase (Raimi *et al.*, 2012). Alkaline phosphatase purified from the hepatopancreas of *Penaeus japonicus* was stable at 65 °C for 5 min. MW of alkaline phosphatase was approximately 40 kDa and the phosphatase from shrimp had an isoelectric point of 7.6 (Chuang and Shih, 1990).

1.2.2 Partitioning systems

1.2.2.1 Aqueous two-phase system (ATPS)

ATPS is a selective method used for biomolecule purification (da Silva and Franco, 2000). ATPS is formed when hydrophilic solutes (polymer or polymer and certain salts) display incompatibility in aqueous solution above critical concentrations (Rito-Palomares, 2004). ATPS offers many advantages including low-process time, low-material cost, low-energy consumption, good resolution, high yield and a relative high capacity. In addition, this system is easily scaled-up (Ruiz-Ruiz *et al.*, 2012). Therefore, ATPS can be applied in the industrial-scale purification of proteins from biomass (Klomklao *et al.*, 2005). The use of ATPS in downstream processing has been focused on the extraction, separation and concentration of various enzymes, including papain (Nitsawang *et al.*, 2006), proteinase (Klomklao *et al.*, 2005), trypsin (Pérez *et al.*, 2015), etc. as well as biomolecules such as amino acid (Du *et al.*, 2007), etc. However, it is sometimes used as a potential primary purification technique to reduce the bulk of processing stream. It can be followed by more selective purification steps, such as chromatography, electrophoresis, etc. (Klomklao *et al.*, 2005).

ATPS of polymer/salt is relatively cheap to ATPS of polymer/polymer (only 1/3-1/5 cost of polymer/polymer). This kind of ATPS therefore has wide application in biotechnology, especially for enzyme separation (Table 2). Among polymer/salt systems, PEG-phosphate is particularly preferred due to several advantages, including low cost, low viscosity (101~102 mPa•s), rapid phase splitting and a wide range of applications (Rosa *et al.*, 2009). The practical ATPS separation strategies can be divided into four main stages: (i) initial physicochemical characterization of the target proteins solution; (ii) selection of the type of ATPS (types of polymers or salts forming ATPS); (iii) selection of system parameters (MW of the polymer, volume ratio and pH); and (iv) evaluation of the influence of process parameters (sample load, neutral salts addition, consecutive extraction stages) upon product recovery/purity (Benavides *et al.*, 2008).

1.2.2.1.1 Formation of aqueous two-phase system

ATPS is formed when two incompatible polymers or one polymer and an inorganic salt are mixed in water above certain critical concentration (Oliveira *et al.*, 2002). Proteins are partitioned between two phases with a partition coefficient that can be modified by changing the medium experimental condition such as pH, salts, ionic strength, etc (Spelzini *et al.*, 2005). Three types of ATPS (i.e. polymer-salt, polymer-polymer and others) have been traditionally used (Table 2). In polymer-salt systems, polyethylene-glycol (PEG)-phosphate ATPS is stable (Rito-Palomares, 2004). In these extraction systems, the product of interest is concentrated in one of the phase forming components, in which the majority of the cases is PEG (Srinivas *et al.*, 1999).

Table 2. Enzymes separated by APTS (Polymer-salt)

Polymer-salt	Enzymes	Sources	Product recovery (%)	References
30% PEG 6000-30% K_2HPO_4	Chymosin Pepsin	*Aspergilus niger* Bovine stomach	81-95 74-93	Spelzini *et al.* (2005)
20% PEG1000–15% $MgSO_4$	Proteinase	Yellowfin tuna	83.6	Klomklao *et al.* (2005)
12% PEG 6000–15% $(NH_4)_2SO_4$	Papain	Wet *Carica papaya* latex	86.2	Nitsawang *et al.* (2006)
22% PEG 10000–8% $(NH_4)_2SO_4$	Proteases	*Clostridium perfringens*	131	Porto *et al.* (2008)
15% PEG2000–15% $MgSO_4$	Proteases	Albacore tuna stomach	89.1	Nalinanon *et al.* (2009)
15% PEG2000–14% $MgSO_4$	Bromelain	pineapple peel	113.5	Ketnawa *et al.* (2010)
12%PEG 3000–14% $MgSO_4$	Protease	*Calotropis procera* latex	65.2	Rawdkuen *et al.* (2011)
42%PEG 400–23% $Na_3C_6H_5O_7$	Lactate dehydrogenase	bovine heart	5.61	de Araújo *et al.* (2011)
15%PEG 3000–23% $MgSO_4$	Invertase	Baker's yeast	217.7	Karkaş and Önal (2012)
18% PEG 1500–15% $(NH_4)_2SO_4$	Pepsinogen	Stomach of red perch	86.2	Zhao *et al.* (2013)
15% PEG2000-15% $Na_3C_6H_5O_7$	Alkaline proteases	Giant catfish viscera	365.5	Ketnawa *et al.* (2013)
40% EOPO 3900-10% MgSO4 with 17%NaCl	Alkaline proteases	Giant catfish viscera	77.98	Ketnawa *et al.* (2014)
10%PEG 6000–10% $(NH_4)_2SO_4$	Glycyl endopeptidase	Papaya latex	98.97	Karnjanapratum and Benjakul (2014)
30% PEG3350-25% $Na_3C_6H_5O_7$	Trypsin	Cow pancrease	99.7	Pérez *et al.* (2015)

The partition coefficients for protein generally fall within the range of 0.1 to 10 (Gu, 2000). For large molecular (such as high MW DNA and RNA) and particles (such as cell and viral particles), partition coefficients > 100 to < 0.01 are observed (Gu, 2000). Small ions tend to partition equally between two phases (Gu, 2000). Partition coefficients (or ratios) of two substances differ, depending on MW of the polymer, pH, the presence of salts and ions in the medium, macromolecular hydrophobicity, etc. (Spelzini *et al.*, 2005).

1.2.2.1.2 Factors affecting partitioning systems

- Effect of polymer, MW and concentration

MW of polymers strongly affects the partitioning of the protein in ATPS (Saravanan *et al.*, 2007). Kula (1979) proposed that if it is desirable to have a higher partition coefficient, lowering the average PEG MW may help. The effect of polymer MW depends on MW of the solute (Saravanan *et al.*, 2007). Polymers with higher MW and higher concentration usually bring higher viscosities to the liquid solutions (Gu, 2000). MW and concentration of PEG have significant effects on protein partition. The extraction with low MW PEG4000 showed the best condition for partition tannery wastewater protein in PEG + $MgSO_4$ + water system (Saravanan *et al.*, 2007). Spelzini *et al.* (2005) found that the increase of PEG concentration for partitioning of pepsin and chymotrypsin favored the protein transfer to the top phase. Klomklao *et al.* (2005) revealed that phase composition including PEG MW and concentration as well as types and concentration of salt affected the partition of proteinase from the spleen of yellowfin tuna (*Thunnas albacores*).

- Effect of temperature

Temparature affects the phase separation. When temperature is decreased, phase separation occurs at lower polymer concentration for PEG-dextran-water systems. Thus optimal temperature of PEG and dextran system is needed to achieve phase separation (Gu, 2000). Temperature also affected PEG-salt-water systems (Lu *et al.*, 1994). Partition coefficients of lysozyme and catalase in different PEG-dextran-water systems were observed at different temperatures (Zaslavsky *et al.*,

1982). A lower temperature causes the viscosity to be higher. Room temperature can be used with minimal bioactivity losses. Therefore, chilling aqueous two phase systems is usually not required, unless very fragile proteins are involved (Gu, 2000).

- Effect of salt

Salts at moderate concentrations have the effects on the phase separation of nonionic polymer-polymer-water systems (Gu, 2000). Usually a much lower polymer concentration is required for phase separation when the salt concentration increases (Gu, 2000). Salt can be used rather effectively to change the partition coefficient of biomolecules. At low salt concentration (0.1 to 0.2 M), the effects of salt type and concentration can be dramatic for protein at pH far always from their isoelectric point (Gu, 2000). As rule of thumb, the decrease of partition coefficient for negatively charged proteins in PEG-dextran-water system is sulfate > fluoride > acetate > chloride > bromide > iodide and lithium > ammonium > sodium > potassium. Positively charged protein follows the opposite trend (Nalinanon *et al.*, 2009). Albertsson *et al.* (1987) reported that increasing NaCl concentration in the range of 0 to 5 M greatly increased the partition coefficient of several proteins (phycocyanin, phycoerythrin, gamma globulin, ceruloplasmin, and serum albumin) in a phase system containing 4.4% PEG8000 and 7% dextran at pH 6.8.

1.2.2.1.3 Partitioning and recovery of proteases by ATPS

ATPS has been used successfully for partitioning and recovery of some proteases. Klomklao *et al.* (2005) demonstrated the partitioning of spleen proteinase from yellowfin tuna using an ATPS. ATPS comprising PEG1000 (15%, w/w) and magnesium sulfate (20%, w/w) provided the best condition for the maximum partitioning of the proteinase into the top phase and gave a highest specific activity (47.0 units/mg protein) and purification fold (6.61). The yield of 69.0% was obtained. Under the same ATPS condition used, the partitioning of proteinase of splenic extract from three tuna species involving skipjack tuna, yellowfin tuna and tongol tuna were compared. The purity of splenic extract from all tuna species increased after ATPS process. Among all species tested, yellowfin tuna showed the highest purification

fold, followed by tongol tuna and skipjack tuna, respectively. SDS-substrate gel electrophoresis revealed that the band intensity of major proteinase in ATPS fraction from all tuna species slightly increased with the concomitant decrease in band intensity of other contaminating proteins, indicating the greater specific activity of splenic extract. Spelzini *et al.* (2005) found that the partition performed at pH 7.0 showed high affinity of chymosin and pepsin for the PEG rich phase. The increase of PEG concentration favored the protein transfer to the top phase, suggesting an important protein–polymer interaction. PEG shows a stabilizing effect on the chymosin and pepsin (Spelzini *et al.*, 2005). The partition behavior of trypsin in poly (ethylene glycol) (PEG)-cashew-nut tree gum aqueous two-phase systems were studied by Oliveira *et al.* (2002). The system composition had little effect on trypsin partition coefficients. In some cases, the NaCl addition changed dramatically the partition coefficient. Altering the conditions allows the manipulation of the protein partition (Oliveira *et al.*, 2002). The maximum recovery of trypsin activity in the cashew-nut tree gum phase was obtained with ATPS containing PEG (MW 8000) and 1.0 M NaCl at pH 7.0. The partitioning of bovine trypsin and α-chymotrypsin, proteases of similar physico-chemical properties, in different PEG/sodium citrate ATPS was also investigated (Tubío *et al.*, 2007). Both a decrease in PEG MW and an increase in pH led to a higher partition coefficient for both enzymes. Tubío *et al.* (2007) revealed that ATPS formed by PEG of MW 3350 and citrate pH 5.2 shows the best separation capability which was enhanced in the presence of 3% NaCl. The transfer of both proteins to the top phase was associated with negative enthalpic and entropic changes. Spelzini *et al.* (2008) found that PEG1450 had a higher interaction capacity with the protein than PEG8000. PEG1450 showed interpolymer interaction, leading to the complex formation, whereas PEG8000 exhibited a cooperative interaction between the polymer and protein molecules which was independent of the PEG concentration. ATPS of trypsin from bovine pancreas in PEG (30% PEG3350) combined with citrate (25% $Na_3C_6H_5O_7$) and NaCl at the concentration of 3.37% had a purification factor of 2.55 and a yield of 99.7% (Pérez *et al.*, 2015).

1.2.2.2 Three-phase partitioning (TPP)

TPP is a method which may be used for the fractionation and concentration of proteins. *t*-Butanol is infinitely miscible with water, but can be induced to form a separate phase by the addition of salt. If protein is present in the solution, it may form a third phase, between the aqueous and *t*-butanol phases, consisting of concentrated and dewatered protein. A claimed advantage of the method is that protein complexing agents, such as tannins and phenolics, and other enzyme inhibitors may be extracted into the phase partition, thus enhancing the purification of proteins from plants and micro-organisms. Lipids would be similarly extracted (Pike and Dennison, 1989). The method is useful both upstream with crude samples and downstream where a saleable simple step is needed. Upon the addition of enough salt, such as ammonium sulfate, the solution separates into two phases, a lower aqueous phase and an upper, *t*-butanol phase. If protein is present in the original aqueous phase, it may, depending on the concentration of ammonium sulfate added, separate into a third phase, intermediate between the lower aqueous and upper *t*-butanol phase (Dennison and Lovrien, 1997).

TPP was first developed as an ‘‘upstream’’ technique for liter scale precipitation of crude cellulases (Dennison and Lovrien, 1997) and other enzymes (Lovrien *et al.*, 1987). However, TPP is frequently also useful downstream for isolation on a semimicro, milliliter volume scale, shown in Figure. 1. TPP development has one root in 1972 work (Tan and Lovrien, 1972) which showed that numbers of enzymes maintain their activities in *t*-butanol–water mixtures. Some enzymes have enhanced catalytic activities in *t*-butanol–water. A few enzymes and proteins lose activity or function in such cosolvent–water mixtures. On the other hand, it can also be useful if unwanted proteins can be shunted aside, such as hemoglobin, in isolating erythrocyte enzymes via the TPP technique (Pol *et al.*, 1990). TPP is related to conventional salting out, not simply because TPP uses the same salt, ammonium sulfate, but because TPP and conventional salting out have some common origins. Sulfate is the leading Hofmeister kosmotrope and anionic ‘‘water structure promoter’’ (Collins and Washabaugh, 1985). By a number of criteria described by Rachana and Lyju Jose (2014), *t*-butanol in water is also a powerful structure

promoter. Although sulfate, *t*-butanol in water is an anion and *t*-butanol is a neutral molecule, it is clear now that such pairs of compounds used in adequately large concentrations reinforce each other physicochemical behavior when the principal solvent is water.

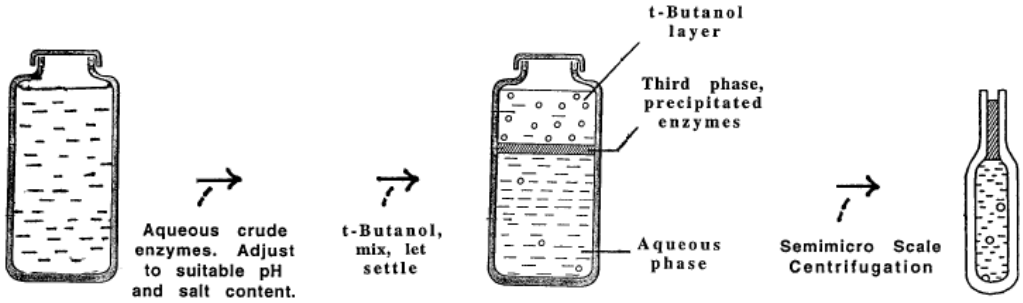

Figure.1 TPP general method, macroscale and semimicro scale. *t*-butanol amounts needed to form an independent second phase and protein precipitate (third phase): from 0.2 to 0.5 ml *t*-butanol per milliliter aqueous phase depending on salt concentration. The two liquid phases extract unwanted materials from crude protein samples.

Source: Dennison and Lovrien (1997)

1.2.2.2.1 Formation of TPP and applications

TPP uses *t*-butanol and ammonium sulfate to precipitate enzymes and proteins from aqueous solutions (Pol *et al.*, 1990). The protein may be compressed into a pellet by relatively slow-speed centrifugation. Such pellets are largely free of salts and contain modest amounts of water and some *t*-butanol. For conventional salting out with ammonium sulfate, the extent of protein precipitation in TPP is a function of the ammonium sulfate concentration. Contaminants preferentially soluble in organic solvents such as lipids are removed in the *t*-butanol layer. A useful feature of TPP–*t*-butanol, then, is that, besides partitioning and concentrating proteins, it sometimes extracts unwanted lower MW compounds such as lipids, phenolic compounds, and somedetergents. Formation of the precipitated protein midlayer usually occurs in a few minutes (Dennison and Lovrien, 1997). TTP to separate several enzyme and inhibitor are summarized in Table 3.

Table 3. Enzymes and inhibitor separated by *t*-Butanol-TPP

Enzymes/inhibitor	Sources	% Salt (w/v)	Ratio of salt: solvent (v/v)	pH	Fold purification	Yield (%)	References
Phospholipase D	*Dacus carota* carrot	30 % $(NH_4)_2SO_4$	1:1	7.0	13	72	Sharma and Gupta (2001c)
Protease/amylase inhibitor	wheat germ	30 % $(NH_4)_2SO_4$	1:1	7.0	25	85	Sharma and Gupta (2001b)
Pectinases	*Aspergillus niger*	30 % $(NH_4)_2SO_4$	1:1	5.0	9	76	Sharma and Gupta (2001a)
Xylanase	*Aspergillus niger*	30 % $(NH_4)_2SO_4$	1:1	4.5	95	60	Sharma and Gupta (2002)
Green fluorescent protein	*E. coli* DH5	30 % $(NH_4)_2SO_4$	1:1	7.0	23	56	Jain *et al.* (2004)
Xylanase	*Aspergillus niger*	30% $(NH_4)_2SO_4$	1:1	5.6	21	93	Roy *et al.* (2004)
Peroxidase	*Ipomoea palmate* leaves	30 % $(NH_4)_2SO_4$	1:1	9.0	18	81	Narayan *et al.* (2008)
Invertase *β*-galactosidase	*Aspergillus oryzae* *Aspergillus oryzae*	30% $(NH_4)_2SO_4$ 30% $(NH_4)_2SO_4$	1:1 1:1	5.0 4.8	5.2 12	54 92	Dhananjay and Mulimani (2008)
Exo-polygalacturonase	*Aspergillus sojae*	30% $(NH_4)_2SO_4$	1.1	6.6	-	25.5	Dogan and Tari (2008)
Trypsin inhibitor	Adzuki bean Navy bean Red kidney bean	30% $(NH_4)_2SO_4$ 30% $(NH_4)_2SO_4$ 30% $(NH_4)_2SO_4$	1:1 1:1 1:1	8.2 8.2 8.2	23.2 4.8 9.5	28 160 398	Wati *et al.* (2009)
β galactosidase	*Aspergillus oryzae*	60 % $(NH_4)_2SO_4$	1:1	4.8	12	92	Dhananjay and Mulimani (2009)
Protease	Papaya peels	20 % $(NH_4)_2SO_4$	1:0.5	7.0	23.8	165.2	Chaiwut *et al.* (2010)
Protease	*Calotropis procera* latex	30% $(NH_4)_2SO_4$	1:0.5	8.0	6.9	132	Rawdkuen *et al.* (2010)
Protease inhibitor amylase inhibitor	Ragi (*Eleusine coracana*)	30% $(NH_4)_2SO_4$ 30% $(NH_4)_2SO_4$	1.1 1.1	6.9	16.3 20.16	31.5 39.5	Saxena *et al.* (2010)
Exo-Inulinase	Inulin	30 % $(NH_4)_2SO_4$	1:0.5	4.6	10.2	86	Vinoth Kumar *et al.* (2011)
Protease	Farmed giant catfish	50 % $(NH_4)_2SO_4$	1:0.5	8.0	5.0	163.1	Rawdkuen *et al.* (2012)
α-Galactosidase	Watermelon	50 % $(NH_4)_2SO_4$	1:1	5.5	2.7	76.7	Bayraktar and Önal (2013)
Ficin	Mediterranean fig latex	40 % $(NH_4)_2SO_4$	1:0.75	6.5	6.04	167	Gagaoua *et al.* (2014)
Nattokinase	Fermentation broth of *Bacillus natto* NRRL-3666	30 % $(NH_4)_2SO_4$	1:1.5	8.0	5.6	129.5	Garg and Thorat (2014)
β-Amylase	Stems of *Abrus precatorius*	49.46 % $(NH_4)_2SO_4$	0.87:0.87	10.17	5.6	156.2	Sagu *et al.* (2015)

1.2.3 Compositions of crustacean processing byproducts

1.2.3.1 Proteins and amino acid

Crustacean processing byproducts are the important source of protein and amino acids. It contained protein in the range of 40-43% (Nwanna *et al.*, 2004). Generally, the shrimp processing byproducts including head shell, etc. had a high content of essential amino acids, indicating a high nutritional value used for food and animal feed (Gildberg and Stenberg, 2001). López-Cervantes *et al.* (2006) reported that free amino acid contents in the fermented shrimp waste ranged from 9.3 to 56.9 mg/g dry mass. The sample contained arginine, histidine, isoleucine, leucine, lysine, methionine, phenylalanine, threonine, tryptophan, valine, asparagine, serine, glutamine, glycine, alanine and proline. Tyrosine was the most abundant amino acid (56.9%). Oyedapo A (1996) produced shrimp head silage by fermenting heads of river prawn (*Macrobrachium vollenhovenii*) with *Lactobacillus plantarum* at 30°C for 7 days and found that protein content of the silage was 40-50%. The silage consisted of arginine, histidine, isoleucine, leucine, lysine, methionine, phenylalanine, threonine, tryptophan and valine.

1.2.3.2 Lipids and fatty acids

Lipids are one of major components in crustaceans. Lipid contents vary with crustacean species. Total lipid in shellfish including mollusks and crustacean is usually 1 to 3% (Karakoltsidis *et al.*, 1995). Shrimp hepatopancreas has been known as the excellent source of lipid and carotenoid, etc. (Takeungwongtrakul *et al.*, 2012). Krill lipid is rich in long chain n-3 polyunsaturated fatty acids (PUFA), mainly docosahexaenoic acid (DHA) and eicosapentaenoic acid (EPA), which have been associated with a wide-range of health benefits to humans (Burri *et al.*, 2012). Lipid compositions of krill from the Scotia Sea and krill from the Gerlache Strait include phosphatidylcholine (PC) (33–36%), phosphatidylethanolamine (PE) (5–6%), triacylglycerol (TG) (33–40%), free fatty acids (FFA) (8–16%) and sterols (1.4–1.7%) (Fricke *et al.*, 1984). Bhavan *et al.* (2010) reported that ω 6 fatty acids were dominant in fresh water shrimp, while ω 3 fatty acids were prevalent in marine shrimp. Lipids

of shrimp head and shell from Indian white shrimp (*Penaeus indicus*) were extracted with the yield of 9.8% (dry weight basis) (Ravichandran *et al.*, 2009). Amiguet *et al.* (2012) reported that shrimp lipid extracted from Northern shrimp (*Pandalus borealis Kreyer*) processing by-products using supercritical CO_2 extraction at 35 MPa and 40 °C had red color and was rich in ω-3 PUFAs (7.8 ± 0.06% EPA and 8.0 ± 0.07 % DHA). Takeungwongtrakul *et al.*, (2012) reported that the lipids from cephalothorax of Pacific white shrimp had higher contents of both DHA and EPA than those from hepatopancreas. Lipids from cephalothorax had EPA and DHA contents of 4.65 and 8.34 g/100 g lipids, respectively, whereas EPA and DHA at levels of 2.15 and 6.20 g/100 g lipids, respectively, were found in lipids from hepatopancreas. Krill oil was also extracted from krill, a shrimp-like crustacean (Sampalis *et al.,* 2003). Similar to fish oil, krill oil is rich in long chain n-3 polyunsaturated fatty acids, mainly DHA and EPA (Duan *et al*., 2010). Furthermore, krill oil contains vitamin E, vitamin A, vitamin D and canthaxanthin. The antioxidant potency of krill oil was found to be 48 fold more potent than fish oil (Sampalis *et al.*, 2003). Krill oil extracted from raw krill meal (*Euphausia supe*) using hexane contained 49.30% triglycerides, 44.16% phospholipids, 1.45% free fatty acids and 4.69% cholesterols. While, polyunsaturated fatty acids (PUFA), monounsaturated fatty acids (MUFA) and saturated fatty acids (SFA) accounted for 20.89%, 37.84% and 41.27% of the total fatty acids, respectively (Yin *et al.*, 2014).

1.2.3.3 Carotenoid and carotenoproteins

Body color of crustaceans is the one of the important factors that affects their commercial value. Astaxanthin is a red-orange carotenoid found in fish and shellfish. It is mainly associated with the color of invertebrate animals such as shrimp, crabs, and lobsters. Generally, it presents in crustaceans as a protein-pigment complex (Armenta-López *et al.*, 2002). Astaxanthin is the predominant carotenoid in penaeids (Okada and Yamaguchi, 1994).Three forms of astaxanthin, namely diester, monoester, and free, were found in black tiger shrimp (Okada and Yamaguchi, 1994). The main carotenoid in the carotenoprotein was free astaxanthin, and no significant difference was found between astaxanthin diester and astaxanthin monoester (Okada and Yamaguchi, 1994). This complex can be green, purple, or blue in the living

animal, acquiring a red color when subjected to heat treatment (Armenta-López *et al.*, 2002). The red color of cooked crustaceans appears by the release of the individual astaxanthin prosthetic groups from the carotenoproteins when denatured by heat (Lorenz, 1998). Astaxanthin in crustaceans are mostly esterified to fatty acids (Armenta and Guerrero-Legarreta, 2009). Guillou *et al.* (1995) reported that astaxanthinin in shrimp (*Pandalus borealis*) was presented as diester, monoester and free forms (76%, 20% and 4%, respectively, relative to total astaxanthin).

Carotenoids as well as carotenoproteins are responsible for the color of crustaceans. Since crustaceans are unable to synthesize carotenoids, astaxanthin or appropriate precursors must be supplied in the diet (Liñán-Cabello *et al.*, 2002). In nature, protein-pigment interaction increases carotenoid stability (Armenta-López *et al.*, 2002). The use of proteolytic enzymes to disrupt the protein-pigment bond increased carotenoid extraction by 58% (Cano-Lopez *et al.*, 1987) or spitted the chitin-pigment interaction, resulting in protein enriched pigments (Armenta-López *et al.*, 2002). Carotenoids can be extracted using traditional solvent extraction, supercritical fluid extraction and vegetable oils (Sowmya and Sachindra, 2012).

The carotenoid content in Indian shrimp processing byproducts was found to vary from 35 to 153 µg/g depending on the species and the major pigments were astaxanthin and its esters (Sachindra *et al.*, 2005). The recovery of carotenoproteins from black tiger shrimp shells was maximized by the hydrolysis of shrimp shells using trypsin from bluefish (1.2 trypsin units/g shrimp shells) for 1 h at 25 ºC (Klomklao *et al.*, 2009). Carotenoprotein recovered contained 19.76% lipid and 87.91 mg total astaxanthin/g sample (Klomklao *et al.*, 2009). Armenta and Guerrero-Legarreta (2009) reported that carotenoproteins from fermented Pacific white shrimp waste were hydrolyzed with a combination of protease and lipase. Carotenoproteins from shrimp heads were recovered by autolysis at 50°C and pH 8.0. Highest carotenoid content in carotenoprotein was reported when autolysis was conducted for 4 h (Sowmya and Sachindra, 2012). Carotenoid extracts from shrimp (*Penaeus indicus*) processing discards were evaluated for antioxidant activity. Crude extract and fractions rich in astaxanthin showed strong antioxidant activity as indicated by radical scavenging, reducing activity and metal chelating activity, comparable to that of the known antioxidants α-tocopherol and TBHQ. Singlet oxygen quenching activity and

nitric oxide scavenging activity of crude extract and its fractions was higher than that of α-tocopherol (Sowmya and Sachindra, 2012). Sachindra *et al.* (2006) extracted shrimp waste carotenoids using different organic solvents and solvent mixtures. A 50:50 mixture of isopropyl alcohol and hexane gave the highest (43.9lg/g waste) carotenoid extraction yield, compared to acetone, methanol, ethanol, isopropyl alcohol, ethyl acetate, ethyl methyl ketone, petroleum ether, and hexane individually and to a mixture of acetone and hexane. Parjikolaei *et al.* (2015) reported that the extraction of astaxanthin from shrimp processing byproducts by green solvents (sunflower oil and methyl ester of sunflower oil). The highest astaxanthin content was achieved at a temperature of 70 °C, using a solvent to waste ratio of 9 and a stirring speed of 400 rpm. Sunflower oil could extract astaxanthin with the yield of 80% and 60%, relative to total astaxanthin extracted by organic solvents. Protein-free astaxanthin can be used in a variety of products such as foods, feeds, cosmetics, and pharmaceuticals.

1.2.4 Fish protein hydrolysate

Fish protein hydrolysate can be produced from fish meat, byproducts of fish processing, etc. Generally, lean fish are ideal for fish protein hydrolysates preparation. Nevertheless, dark fleshed fish containing high amount of lipid and heme proteins, could be also used for production of protein hydrolysates (Klompong *et al.*, 2007). Additional treatments such as washing, defatting or centrifugation in order to remove the fat and pigments are required for better quality, acceptability and stability. The use of washed mince or surimi generally gives the high quality fish protein hydrolysates (Kristinsson and Rasco, 2000).

1.2.4.1 Enzymatic hydrolysis

Cleavage of proteins into smaller molecules or peptides during enzymatic hydrolysis process could improve the functional and nutritional properties of food proteins (Kudo *et al.*, 2009). Enzymatic hydrolysis can be done via proteolytic enzymes, both endopeptidases which cleave the peptide bonds within protein molecules and exopeptidases which hydrolyze the terminal peptide bonds (Benjakul

and Morrissey, 1997). Proteases have been used widely to obtain a more selective hydrolysis since they are specific for peptide bonds adjacent to certain amino acid residues (Rao *et al.*, 1998).

A wide variety of commercial enzymes has been used successfully to hydrolyze fish proteins (Table 4). The selection of enzymes is usually based on a combination of efficacy and economics (Lahl and Braun, 1994). In comparison to animal- or plant-derived enzymes, microbial enzymes offer several advantages including a wide variety of available catalytic activities, greater pH and temperature stabilities (Guerard *et al.*, 2002). Proteolytic enzymes from microorganisms such as Alcalase (Ovissipour *et al.*, 2013; Silva *et al.*, 2014), Flavourzyme (Elavarasan *et al.*, 2014; Ovissipour *et al.*, 2013), Neutrase (Chi *et al.*, 2014; Phanturat *et al.*, 2010), Protamex (Pires *et al.*, 2015) and Protease N (Wu *et al.*, 2003) are suitable to prepare fish protein hydrolysates because of their high productivity. Commercial enzymes were also successfully used to produce protein hydrolysates with bioactivities. Silva *et al.* (2014) reported that tilapia processing waste hydrolyzed by Alcalase showed the antioxidative activity. The antioxidative activities were found in protein hydrolysates derived from mackerel prepared by Protease N (Wu *et al.*, 2003). Protein hydrolysate from tilapia and fresh water carp hydrolyzed with Alcalase and Flavourzyme showed the antioxidative activities (Elavarasan *et al.*, 2014; Silva *et al.*, 2014). Additionally, skate (*Okamejei kenojei*) skin gelatin hydrolysate prepared using Alcalase showed ACE inhibitory activity (Ngo *et al.*, 2015). Recently, Chalamaiah *et al.* (2015) used pepsin and trypsin for enzymatic hydrolysis to produce antioxidant peptides from Common carp roe. The combinations of proteinases including Alcalase, Protamex, Flavourzyme, Promod, Papain and Bromelain have also been used to increase the bioactivity of protein hydrolysates from whole anchovy sprat (Ovissipour *et al.*, 2013). Barzideh *et al.* (2014) prepared ACE inhibitory gelatin hydrolysate from ribbon jellyfish using trypsin, Alcalase and Protamex.

Table 4. Commercial proteases used for production of fish protein hydrolysate

Proteases	Substrates	References
Protamex	Atlantic salmon (*Salmo salar*, L.) frames	Liaset *et al.* (2003)
Protease N	Mackerel (*Scomber austriasicus*) meat	Wu *et al.* (2003)
Alcalase	Atlantic salmon (*Salmo salar*) head	Gbogouri *et al.* (2006)
Flavourzyme, Neutrase	Cod (*Gadus morhua*) viscera, backbone	Šližytė *et al.* (2005)
Alcalase	Red salmon (*Oncorhynchus nerka*) head	Sathivel *et al.* (2005)
Alcalase, Flavourzyme	Round scad (*Decapterus maruadsi*) mince	Thiansilakul *et al.* (2007)
Alcalase, Flavourzyme	Yellow stripe trevally (*Selaroides leptolepis*) mince	Klompong *et al.* (2007)
Alcalase, Flavourzyme	Silver carp (*Hypophthalmichthys molitrix*) mince	Dong *et al.* (2008)
Papain	Grass carps (*Ctenopharyngodon idellus*) muscle	Ren *et al.* (2008)
Protamex	Atlantic mackerel (*Scomber scombrus*)	Beaulieu *et al.* (2009)
Orientase	Tuna (*Thunnus tonggol*) cooking juice	Hsu *et al.* (2009)
Papain, Protamex	Loach (*Misgurnus anguillicaudatus*) meat	You *et al.* (2009)
Orientase, Protease XXIII	Tuna (*Thunnus tonggol*) dark muscle	Hsu (2010)
Protamex	Black scabbardfish (*Aphanopus carbo*) heads, viscera, frames, skin, trimmings	Batista *et al.* (2010)
Alcalase, Flavourzyme	Bluewing Searobin (*Prionotus punctatus*) meat	dos Santos *et al.* (2011)
Alcalase, Trypsin, Protamex	Alaska pollock frame muscle	Hou *et al.* (2011)
Alcalase	Threadfin bream (*Nemipterus spp*.) bone, skin	Wiriyaphan *et al.* (2012)
Alcalase	Yellowfin Tuna (*Thunnus albacares*) viscera	Ovissipour *et al.* (2012)
Papain	Scallop (*Patinopecten yessoensis*) meat	Zhou *et al.* (2012a)
Alcalase	Nile tilapia (*Oreochromis niloticus*) muscle	Yarnpakdee *et al.* (2012)
Papain, Pepsin, Trypsin	Pink perch (*Nemipterus japonicus*) muscle	Naqash and Nazeer (2013)
Alcalase, Bromelain, Flavorzyme, Protamex	Fresh Water Carp (*Catla catla*) muscle	Elavarasan *et al.* (2014)
Alcalase	Nile tilapia (*Oreochromis niloticus*) waste	Silva *et al.* (2014)
Protamex	Cape hake (*Merluccius capensis*)	Pires *et al.* (2015)

Apart from commercial protease, fish enzymes have been used to produce protein hydrolysate (Table 5). Fish and shellfish trypsins have been reported as the potential source for the acceleration of protein hydrolysis (Kristinsson and Rasco, 2000). Fish and shellfish trypsins have an advantage over commercial proteases due to the lower costs. The production of protein hydrolysate using fish and shellfish trypsins or fish trypsins in combination with commercial proteases therefore lowers production cost. Trypsin from unicorn leatherjacket (*Aluterus monoceros*) pyloric caeca was used for preparing Indian mackerel muscle protein hydrolysates

containing antioxidative peptides (Zamani and Benjakul, 2015). Antioxidative peptides were also obtained in toothed ponyfish muscle protein hydrolysate prepared using crude protease from hybrid catfish viscera (Klomklao *et al.*, 2013b). Zebra blenny (*Salaria basilisca*) muscle protein hydrolysates with antioxidative activity were prepared with crude alkaline protease from zebra blenny, sardinelle and smooth hound viscera (Ktari *et al.*, 2012a). Additionally, smooth hound muscle hydrolysate prepared using its gastrointestinal proteases possessed the higher antioxidative activities, compared with that prepared using bovine trypsin (Bougatef *et al.*, 2010a).

Table 5. Fish proteases used for production of protein hydrolysate

Proteases	Substrates	References
Tuna pyloric caeca proteases	Cod frame protein	Jeon *et al.*, (1999)
Atlantic salmon proteases	Atlantic salmon (*Salmo salar*) muscle	Kristinsson and Rasco, (2000)
Pepsin, Mackerel intestine proteases	Yellowfin sole (*Limanda aspera*) frame	Jun *et al.*, (2004)
Mackerel intestine proteases	Alaska pollack (*Theragra chalcogramma*) frame	Je *et al.*, (2005)
Smooth hound Intestine protease	Smooth hound (*Mustelus mustelus*) meat	Bougatef *et al.*, (2009)
Cuttlefish hepatopancreas protease	Cuttlefish (*Sepia officinalis*) muscle	Balti *et al.*, (2012)
Sardine viscera protease	Sardinelle (*Sardinella aurita*) heads and viscera	Bougatef *et al.*, (2010b)
Skipjack tuna pepsin	Ornate threadfin bream muscle (*Nemipterus hexodon*) muscle	Nalinanon *et al.*, (2011)
Pyloric caeca of brownstripe red snapper protease	Brownstripe red snapper (*Lutjanus vitta*) muscle	Khantaphant *et al.*, (2011)
Pacific abalone viscera protease	Pacific abalone (*Haliotis discushannai* Ino) viscera	Zhou *et al.*, (2012c)
Yellowfin tuna viscera protease	Yellowfin tuna viscera muscle	Ovissipour *et al.*, (2012)
Crude alkaline protease from zebra blenny, sardinelle and smooth hound viscera.	Zebra blenny (*Salaria basilisca*) muscle	Ktari *et al.*, (2012a)
Hybrid catfish viscera protease	toothed ponyfish (*Gazza minuta*) muscle	Klomklao *et al.* (2013b)
Zebra blenny viscera protease	Shrimp waste	Ktari *et al.* (2014)
Protease from pyloric caeca of unicorn leatherjacket	Indian mackerel (*Rastrelliger kanagurta*) muscle	Zamani and Benjakul (2015)

1.2.4.2 Protein hydrolysates from fish muscle

Muscle of different fish species have been used for the production of protein hydrolysate such as capelin (*Mullotus vilhus*) (Amarowicz and Shahidi, 1997), Atlantic salmon (*Salmo salar*) (Kristinsson and Rasco, 2000), mackerel (*Scomber austriasicus*) (Wu *et al.*, 2003), yellow stripe trevally (*Selaroides leptolepis*) (Klompong *et al.*, 2007), red salmon (*Oncorhynchus nerka*) (Sathivel *et al.*, 2005), round scad (*Decapterus maruadsi*) (Thiansilakul *et al.*, 2007), tilapia (*Oreochromis niloticus*) (Raghavan *et al.*, 2008), silver carp (*Hypophthalmichthys molitrix*) (Dong *et al.*, 2008), smooth hound (*Mustelus mustelus*) (Bougatef *et al.*, 2009), loach (*Misgurnus anguillicaudatus*) (You *et al.*, 2009), ornate threadfin bream (*Nemipterus hexodon*) (Nalinanon *et al.*, 2011), croaker (*Otolithes ruber*) (Nazeer *et al.*, 2012), round scad (*Decapterus maruadsi*) (Jiang *et al.*, 2014), whitemouth croaker (*Micropogonias furnieri*) (da Rosa Zavareze *et al.*, 2014), cape hake (*Merluccius capensis*) (Pires *et al.*, 2015) and tuna dark muscle (Kuo-Chiang, 2010).

Prior to protein hydrolysis, some treatments, especially defatting or concentrating of the protein, have been employed to obtain the better quality of protein hydrolysate (Clemente, 2000). Yellow stripe trevally, a dark flesh fish, was defatted with isopropanol prior to hydrolysis (Klompong *et al.*, 2007). Defatting process could remove fat in fish muscle effectively by 79% and hence increased protein content from 84.2 to 96.8% (dry basis). Moreover, heme proteins naturally present in fish muscle were able to act as pro-oxidants. Washing is the process used for the removal of these sarcoplasmic proteins and pro-oxidative aqueous components (Sannaveerappa *et al.*, 2007). Myoglobin in ordinary muscle of sardine and mackerel mince was removed by 23 and 75 % via washing process (Chaijan *et al.*, 2004). Thus, washing could lower the heme proteins effectively. In addition, washing not only removed sarcoplasmic proteins, but also concentrated the myofibrillar proteins (Baxter and Skonberg, 2008). Khantaphant *et al.* (2011) also pretreated the meat of brownstripe red snapper (*Lutjanus vitta*) by washing and removing phospholipids prior to hydrolysis using the proteases. The resulting hydrolysate had less fishy odor and flavor. Myofibrillar proteins were extracted as protein isolate prior to enzymatic hydrolysis such as channel catfish (*Ictalurus punctatus*) (Theodore *et al.*, 2008). The

protein isolate might be more preferable for the proteinase used. As a consequence, the hydrolysis could take place at the higher degree, compared with that found in the intact muscle.

1.2.4.3 Protein hydrolysate from fish processing byproducts

By-products obtained from fish processing plant have been used for the preparation of protein hydrolysate with functional properties and bioactivities. By-products (heads, viscera, frames, skin, trimmings) of Nile tilapia (*Oreochromis niloticus*) (You *et al.*, 2014), black scabbard fish (*Aphanopus carbo*) (Batista *et al.*, 2010) and those (head and viscera) from sardinella (*Sardinella aurita*) (Khaled *et al.*, 2012) were used to produce the protein hydrolysate. Frame from yellowfin sole (*Limanda aspera*) (Jun *et al.*, 2004), Alaska pollack (*Theragra chalcogramma*) (Je *et al.*, 2005) and hoki (*Joohnius belengerii*) (Kim *et al.*, 2007) as well as backbones from tuna (Je *et al.*, 2007), Atlantic cod (*Gadus morhua*) (Šližytė *et al.*, 2005) and cobia (*Rachycentron canadum*) (Amiza *et al.*, 2014) have been used for preparation of protein hydrolysate. Apart from solid byproducts, liquid effluent such as cooking juice from tuna (Hung *et al.*, 2014; Kasiwut *et al.*, 2014) has been used as the raw material for hydrolysate production. Protein hydrolysate from shrimp head (*Penaeus kerathurus*) using commercial trypsin (0.1%) was rich in protein and had the improved functional properties. The shrimp head could therefore be considered as an alternative for other proteinaceous source, which can be used in the food and animal feed industry, particularly after hydrolysis process is implemented (Limam *et al.*, 2008). Protein hydrolysates of shrimp waste (*Penaeus monodon* and *Penaeus indicus*) prepared by Alcalase showed antioxidative activity in a dose dependent manner (Dey and Dora, 2014).

Protein hydrolysates were also prepared from gelatin extracted from aquatic animals. Gelatin hydrolysates with antioxidative activity have been produced from skin gelatin of Alaska Pollack (Kim *et al.*, 2001), hoki (Mendis *et al.*, 2005b), cobia (Yang *et al.*, 2008), sole (Giménez *et al.*, 2009), blacktip shark (Kittiphattanabawon *et al.*, 2012), chum salmon (Fu and Zhao, 2013), bluefin leatherjacket (Chi *et al.*, 2014), Nile tilapia (Choonpicharn *et al.*, 2014) and unicorn leatherjacket (Karnjanapratum and Benjakul, 2015). Furthermore, gelatin hydrolysates

were also prepared from the skin of jumbo squid (Mendis *et al.*, 2005b), bullfrog (Qian *et al.*, 2008), squid (Giménez *et al.*, 2009), cuttlefish (Jridi *et al.*, 2014), blue shark (Weng *et al.*, 2014) and (Dang *et al.*, 2015). Those hydrolysates were mainly prepared with the aid of proteolytic enzymes. Since the gelatin can be hydrolyzed at high temperature, the thermal hydrolysis was applied to produce the gelatin hydrolysate from cobia (*Rachycentron canadum*) skins (Yang *et al.*, 2008). Recently, gelatin hydrolysate from gelatin from the sea bream scale with the antioxidative activity was prepared by Akagündüz *et al.* (2014).

For some fish species, the hydrolysis can be maximized by optimization of autolysis mediated by endogenous proteases. Autolysis of fish promotes the hydrolysis process and lowers the cost of enzymes. Pacific hake was hydrolyzed by its endogenous proteases from infected *K. paniformis* which produced cathepsin L-like enzyme. Hence, the production of Pacific hake fish protein hydrolysate could be conducted without adding any commercial enzymes (Samaranayaka and Li-Chan, 2008). However, in term of protein recovery, hydrolysates produced autolytically were considerably lower, compared with that produced by commercial proteases (Shahidi *et al.*, 1995). Anchovy protein was autolyzed at pH 8.5 and 50 ºC. After autolysis processing for 24 h, total nitrogen recovery reached 92.1%. The resulting protein hydrolysates had antioxidant properties (He *et al.*, 2014).

1.2.4.4 Antioxidative activity of fish protein hydrolysates

Proteins from marine sources possess specific biological properties after hydrolysis using various proteases (Ahn *et al.*, 2014; Amiza *et al.*, 2014; Chalamaiah *et al.*, 2015; Karnjanapratum and Benjakul, 2015). Fish protein hydrolysates have a potential to be protein supplements in cereal foods and soups (Venugopal and Shahidi, 1998). In some countries, fish protein hydrolysates are used as a milk substitute and as flavoring compounds (Stephens *et al.*, 1976). Additionally, protein hydrolysates have been known to possess antioxidant activity.

Types of proteases have the influence on the peptides produced and their antioxidative activities. Khaled *et al.* (2014) produced protein hydrolysate from

sardinelle (*Sardinella aurita*) muscle using crude enzyme extract from sardinelle viscera. The hydrolysate showed a strong metal chelating activity (89% at 1 mg/ml). Protein hydrolysate from Alaska pollack frame prepared using mackerel intestine crude enzyme also exhibited antioxidative activity in a linoleic acid oxidation system (Je *et al.*, 2005). Toothed ponyfish (*Gazza minuta*) muscle was hydrolyzed using viscera extract from hybrid catfish and the hydrolysate obtained displayed DPPH, ABTS radical scavenging activities, ferric reducing power and metal chelating activity (Klomklao *et al.*, 2013a). Kim *et al.* (2007) prepared antioxidative peptide from hoki frame using different six proteases (pepsin, trypsin, papain, α-chymotrypsin, Alcalase and Neutrase). Peptic hydrolysate showed the highest inhibition against linoleic acid oxidation as well as radical scavenging activities. Alaska pollack skin gelatin was hydrolyzed with Alcalase, Pronase E, and collagenase and the hydrolysate prepared using Pronase E exhibited the highest antioxidative activity (Kim *et al.*, 2001). Furthermore, Batista *et al.* (2010) produced protein hydrolysate from black scabbardfish by-products using Protamex. The resulting hydrolysate possessed DPPH and hydroxyl radical scavenging activities and reducing power. Different proteases exhibited the varying specificity and reaction rate in hydrolysis of peptide chains, resulting in the different peptides with various bioactivities. Phanturat *et al.* (2010) used the pyloric caeca extract from bigeye snapper (*Priacanthus macracanthus*) for preparation of gelatin hydrolysate with antioxidative activity. The antioxidative peptide of gelatin hydrolysate had MW of 1.7 kDa. Protein hydrolysate from defatted skipjack (*Katsuwonous pelamis*) roe using Alcalase 2.4 L had antioxidant activity, both radical scavenging activity and metal chelating activity. The hydrolysates had high solubility and interfacial properties. The hydrolysate with 5%DH exhibited the highest antioxidative and functional properties and it contained two major peptides with MW of 57.8 and 5.5 kDa (Intarasirisawat *et al.*, 2012).

Fish protein hydrolysates normally contain peptide with 2–20 amino acids (Chalamaiah *et al.*, 2012). The variation in amino acid composition of different fish protein hydrolysates mainly depends on several factors such as raw material, enzyme source, and hydrolysis conditions. Among all the amino acids, aspartic acid and glutamic acid were found to be higher in most of fish protein hydrolysates

(Chalamaiah *et al.*, 2012). Fish protein hydrolysate was prepared from tilapia muscle using Alcalase. Glutamic acid, aspartic acid and lysine were the most abundant amino acids present in hydrolysate with values 42.68, 29.16 and 26.21 mg/g, respectively (Roslan *et al.*, 2014). The amino acid composition and sequence as well as the chain length are the factors governing the antioxidative activity of peptides. With the same raw materials, the different proteases used render the peptides with different characteristics, especially bioactivity. To enhance the bioactivity of peptides produced, the several approaches have been implemented such as the use of multi-step of hydrolysis with different proteases (Phanturat *et al.*, 2010). Types of proteases used also showed the impact on the bioactivity of peptides formed (Khantaphant *et al.*, 2011; Phanturat *et al.*, 2010). Different radical scavenging activity is not determined by only amino acid composition but also amino acid sequences (Mendis *et al.*, 2005b). Antioxidant peptides derived from different sources exhibited varying potencies to scavenge free radicals (Table 6). The antioxidant activity may not be attributed to a single antioxidant mechanism (Rajapakse *et al.*, 2005). Some peptides are capable of chelating metal, which acts as the pro-oxidant (Klompong *et al.*, 2007). Wang *et al.* (2009) stated that peptides, which exhibited good antioxidative activity usually, contained certain amino acids such as histidine, proline, tyrosine and lysine. Mendis *et al.* (2005b) reported that the presence of non-aromatic amino acids such as proline, alanine, valine and leucine in jumbo squid skin hydrolysate contributed to the higher antioxidative activities and phenylalanine and leucine residues at N- and C-terminal of peptide could contribute to the high activity. Guo *et al.* (2009) concluded that peptides containing tyrosine residues at the C-terminus, lysine or phenylalanine residues at the N-terminus and tyrosine residues in their sequences had strong free radical scavenging activity. Peptides containing histidine, tryptophan and tyrosine residues possessed antioxidative activity (Bougatef *et al.*, 2010b). Moreover, Suetsuna (2000) indicated that some other amino acids such as proline, alanine and leucine contribute to free radical scavenging activity. Leucine and proline could favor antioxidant activity when it is located at C-terminus of the sequence (Suetsuna, 2000). Antioxidative peptides from different hydrolysates prepared using protease are shown in Table 6.

Table 6. Amino acid sequence and MW of antioxidative peptides from some fish protein hydrolysates

Sources	Enzymes	MW (Da)	Sequences	References
Tuna cooking juice	Protease XXIII	751	Pro-His-His-Ala-Asp-Ser	Jao and Ko (2002)
Yellowfin sole frame	Pepsin, Mackerel intestine crude enzyme	1300	Arg-Pro-Asp-Phe-Asp-Leu-Glu-Pro-Pro-Tyr	Jun *et al.* (2004)
Alaska pollack frame	Mackerel intestine crude enzyme	672	Leu-Pro-His-Ser-Gly-Tyr	Je *et al.* (2005)
Jumbo squid skin gelatin	Trypsin	880	Phe-Asp-Ser-Gly-Pro-Ala-Gly-Val-Leu	Mendis *et al.* (2005a)
		1242	Asn-Gly-Pro-Leu-Gln-Ala-Gly-Gln-Pro-Gly-Glu-Arg	
Hoki skin gelatin	Trypsin	797	His-Gly-Pro-Leu-Gly-Pro-Leu	Mendis *et al.* (2005b)
Hoki frame	Pepsin	1801	Glu-Ser-Thr-Val-Pro-Glu-Arg-Thr-His-Pro-Ala-Cys-Pro-Asp-Phe-Asn	Kim *et al.* (2007)
Bigeye tuna dark muscle	Pepsin	1222	Leu-Asn-Leu-Pro-Thr-Ala-Val-Tyr-Met-Val-Thr	Je *et al.* (2008)
Sardinelle (head, viscera)	sardine viscera crude enzyme	431	Leu-His-Tyr	Bougatef *et al.* (2010b)
Tuna dark muscle by-product	Protease XXIII	756	Pro-Met-Asp-Tyr-Met-Val-Thr	Hsu (2010)
		978	Leu-Pro-Thr-Ser-Glu-Ala-Ala-Lys-Tyr	
Loach muscle	Papain	464	Pro-Ser-Tyr-Val	You *et al.* (2009)
Horse mackerel skin	pepsin, trypsin, α-chymotrypsin	856	Asn-His-Arg-Tyr-Asp-Arg	Kumar *et al.* (2011)
Croaker muscle	trypsin, α-chymotrypsin	861	Lys-Thr-Phe-Cys-Gly-Arg-His	Nazeer *et al.* (2012)
Nile tilapia skin gelatin	Properase E and multifect neutral	317, 645	Glu-Gly-Leu, Tyr-Gly-Asp-Glu-Tyr	Zhang *et al.* (2012)
Nile tilapia muscle	Alcalase	1200	Trp-Glu-Trp-Leu-His-Tyr-Trp	Charoenphun *et al.* (2013)
Salmon byproduct	Alcalase, Flavourzyme, Neutrase, pepsin, Protamex and trypsin	1018	Phe-Leu-Asn-Glu-Phe-Leu-His-Val	Ahn *et al.* (2014)
Croceine croaker muscle	Papain, Alcalase	651, 668, 662	Tyr-Leu-Met-Ser-Arg, Val-Leu-Tyr-Glu-Glu, Met-Ile-Leu-Met-Arg	Chi *et al.* (2015a)
Bluefin leatherjacket heads	Papain	615, 269, 485	Trp-Glu-Gly-Pro-Lys, Gly-Pro-Pro, Gly-Val-Pro-Leu-Thr	(Chi *et al.*, 2015b)

1.2.5 Fish and shellfish lipids

1.2.5.1 Compositions

Lipids are one of major components affecting the quality of fish and shellfish. Total lipid in shellfish including mollusks and crustacean is usually 1 to 2% (Karakoltsidis *et al.*, 1995). Lipids can vary with portions and organ. Lipids of shrimp head and shell from Indian white shrimp (*Penaeus indicus*) were extracted with a yield of 9.8% (dry weight basis) (Ravichandran *et al.*, 2009). Lipid components of shrimp include triglyceride, glycolipid and phospholipids. Among all lipids, phospholipids are the predominant lipid class in crustacean (Yepiz-Plascencia *et al.*, 2000).

Lipid compositions of fish depend on many factors including sex, growth stage and season (Bottino *et al.*, 1980). Fatty acids in fish include 16:0, 16:1ω7, 18:1ω9, 20:5ω3 and 22:6ω3, accounting for 55-90% of total fatty acids (Karakoltsidis *et al.*, 1995). Four essential fatty acids in *Penaeus monodon* included linoleic (18:2n-6, LOA), linolenic (18:3n-3, LNA), eicosapentaenoic (20:5n-3, EPA), and docosahexaenoic (22:6n-3, DHA) acids (Catacutan, 1991). Chanmugam *et al.* (1983) reported that ω6 fatty acids were predominant in fresh water shrimp, while ω3 fatty acids were dominant in marine shrimp. Fatty acid profiles and cholesterol contents of shrimps were reported to change seasonally (Luzia *et al.*, 2003).

By-products or wastes from fish and shellfish processing including heads, frames, and viscera processing are well-known sources of lipids with nutritionally valuable components such as EPA and DHA (Mbatia *et al.*, 2010). Lipid from custacean including krill contains a high amount of phospholipids rich in long chain n-3 PUFA (Bunea *et al.*, 2004). Oil was also extracted from krill, a shrimp-like crustacean (Sampalis *et al.*, 2003). Similar to fish oil, krill oil is rich in long chain ω3 PUFA, mainly DHA and EPA, which have been associated with a wide range of health benefits to humans (Duan *et al.*, 2010). Krill oil, extracted from Antarctic krill, represents a novel source of PUFA. The amount of n-3 PUFAs of whole krill accounted for 19.0 ± 1.7% of fatty acids (Bustos *et al.*, 2003). Environmental and physiological factors affect the level of PUFA in marine lipids. Additionally, the

extraction methods and solvents used also had the impact on fatty acid composition in krill oil (Lambertsen and Braekkan, 1971). Furthermore, krill oil contains vitamin E, vitamin A, vitamin D and canthaxanthin. The antioxidant potency of krill oil was found to be 48 fold higher than fish oil (Sampalis *et al.*, 2003). Lipid composition of krill from the Scotia Sea and krill from the Gerlache Strait were similar. The main lipids were phosphatidylcholine (33–36%), phosphatidylethanolamine (5–6%), triglyceride (33–40%), free fatty acids (8–16%) and sterols (1.4–1.7%) (Fricke *et al.*, 1984).

1.2.5.2 Extraction of lipids

Lipids from fish and shellfish can be produced by several methods, including hexane extraction (Hara and Radin, 1978), supercritical fluid extraction, and heat treatment, which may affect quality and content of PUFAs (Gbogouri *et al.*, 2006). Enzymatic oil extraction using commercial, low cost food grade proteases provides an attractive alternative due to mild condition and short period of time (Linder *et al.*, 2005). Commercial proteases have been used to release oil from marine by-products, resulting in the improved yields as compared to that obtained from heat rendering process (Gbogouri *et al.*, 2006). In addition, the resulting hydrolysate, a byproduct, can serve a good source of soluble fish proteins. However, pH adjustment and addition of water to the reaction medium are implemented. This is not industrially desirable since it adds to the process cost and bulkiness (Mbatia *et al.*, 2010).

1.2.5.2.1 Solvent extraction

Solvent systems have been developed for lipid extraction. Those include chloroform-methanol system, n-hexane-isopropyl alcohol, and methylene chloride-methanol systems. The extraction methods are labor intensive, lack of precision and require a large volume of solvent (Lee *et al.*, 1995). The amount of lipid extracted from tissue depends on the nature of the tissue and the solvent used (Dobush *et al.*, 1985). The solvent used should depend on the type of lipids (Lee *et al.*, 1995), and polarities (Dobush *et al.*, 1985). The widely used methods for extraction of lipids from fish and other marine tissues involve various mixtures of chloroform and

methanol as described by Folch *et al.* (1957) and Bligh and Dyer (1959). Chloroform-methanol is likely the most effective solvent for extracting total fat (Bligh and Dyer, 1959). Nevertheless, chloroform - methanol also may extract some nonlipid materials, e.g., amino acids and carbohydrates (Bligh and Dyer, 1959). Lipids were extracted from yellowfin tuna (*Thunnus albacares*) and bigeye tuna (*Thunnus obesus*) muscle using a chloroform-methanol solution (2:1, v/v). The major fatty acids of both samples were C16:0, C18:1, C22:6 (DHA), and C18:0. Yellowfin tuna had a higher concentration of DHA (20.22%, % of total fatty acids), however, no differences in C20:4 (ARA) and C20:5 (EPA) were found between the two tuna fish. The n-3/n-6 ratios of yellowfin tuna and bigeye tuna were 3.29 and 4.56, respectively (Peng *et al.*, 2013). Shabani Kakroodi *et al.* (2014) reported that fish oil from liver and tissue of patin catfish (*Pangasianodon hypophthalmus*) was extracted using chloroform – methanol mixtures (Bligh and Dyer method). The fat contents of fatty tissue and liver of females were 77.64 and 11.71%, respectively, whereas the contents of 73.23 and 9.59% were found in male, respectively. The major fatty acids presented in these tissues were palmitic (C16:0), oleic (C18:1n-9), and linoleic acid (C18:2 n-6). The total amount of PUFA of liver from male and female patin catfish were 13.31 and 13.30%, respectively, whereas in the fatty tissue these were 11.64 and 12.09%, respectively. The n-3 to n-6 ratios of liver and fatty tissue of females were 1.61 and 0.95, respectively, whereas in male fish, these were 1.31 and 1.05, respectively. The liver and fatty tissues of patin catfish are therefore suitable sources of fish oil, due to the presence of monounsaturated and n-3 PUFAs.

1.2.5.2.2 Supercritical fluid extraction

Extraction with supercritical fluid extraction (SC-CO_2) has been proposed as a good method for obtaining fish oil with a high amount of omega-3 fatty acids. It involves the use of a non-oxidant atmosphere and mild temperatures, which prevent the oxidation of the PUFAs (Rubio-Rodríguez *et al.*, 2012). Rubio-Rodríguez *et al.* (2008) reported that fish oil extracted from hake offcuts using SC-CO_2 at a pressure of 25 MPa and a temperature of 313 K. The highest yield and extraction rate were reached when the by-products were previously cut and freeze-dried to moisture content below 20% in order to improve the oil–SC-CO2 contact and minimize oil–

water interaction in the supercritical phase. Fish oil was extracted from the head of longtail tuna (*Thunnus tonggol*) using various supercritical carbon dioxide (SC-$C0_2$) techniques and the Soxhlet method. The extraction was performed at temperature range of 45 to 65 °C and pressure range of 20-40 MPa, where 65 °C/40 MPa had the highest yield of 20.6, 35.4 and 34.1 g/ 100 g sample (dry basis) for the continuous, cosolvent, and pressure swing techniques of SC-$C0_2$, respectively. The co-solvent method of SC-$C0_2$ was regarded as the most effective method, rendering the highest oil yield with the least amount of $C0_2$ consumption from tuna head wastes (Amiguet *et al.*, 2012).

1.2.5.2.3 The use of proteases

Exogenous proteases have been used to hydrolyze tissue proteins, resulting in the release of oil from the protein matrix of marine by-products (Daukšas *et al.*, 2005). Lipids extracted from salmon (*Salmo salar*) heads by commercial protease had the yield of 19.6%, while the yield of lipids obtained from cooking method was lower (14.5%) (Gbogouri *et al.*, 2006). The mixtures of cod (*Gadus morhua*) containing viscera showed the highest lipid recovery (up to 82.8% of total lipids), compared with those without viscera (Daukšas *et al.*, 2005). Linder *et al.* (2005) evaluated several commercial proteases (Alcalase®, Neutrase® and Flavourzyme®) for their ability to release the oil from salmon heads. The amount of oil (17.4%) obtained after 2 h of hydrolysis with Alcalase 2.4L was close to that obtained by the chemical extraction method (20%) (Linder *et al.*, 2005). Salmon heads hydrolyzed with 0.5% (w/w) bromelain without water addition (1 h, 55 °C) had an oil yield of 11.8 ± 0.4% g lipids/100 g wet weight (Mbatia *et al.*, 2010). Liaset *et al.* (2003) recovered 77% of total lipids from the salmon frames by enzymatic hydrolysis with Protamex®. Fish visceral waste hydrolyzed with fungal protease had the highest recovery of lipids (74.9%) as well as higher DH (49.1%) when compared with hydrolysate using Alcalase (61.7%) (Hathwar *et al.*, 2011). Zhou *et al.* (2012b) reported that enzyme-assisted organic solvent extraction and SC-CO_2 extraction were established to recover lipid from abalone (*Haliotis discus hannai* Ino) gonad. Enzyme-assisted organic solvent resulted in 60.00 ± 2.16% recovery of lipid from abalone gonad digested with neutral protease. In addition, a lipid yield of 82.56 ±

2.77% was achieved by SC-CO_2 extraction. Thus, two lipid-extraction steps can be combined into the comprehensive utilization process of shellfish by-product**.** Furthermore, fish oil was extracted from the mixed solution of tuna oil and water using trypsin. The best extraction conditions were 1.71% trypsin at pH 7.94 and 44 ℃ and 4.22 h. The extraction of fish oil with yield of 90.23% was achieved (Jian-dong, 2012).

1.2.6 Coconut oil

Coconut is another source of oil for food consumption and chemical applications. It is abundant in South East Asia, e.g. Thailand, Malaysia, Philippines and Indonesia which produce major quantities of coconut in the world (Nakpong and Wootthikanokkhan, 2010). Coconut oil is edible oil that has been consumed in tropical countries for thousands of years. It has a long shelf life with a melting point of 76 °F (Marina *et al.*, 2009b). Coconut oil is commercially derived from copra, which is the dried kernel or 'meat' of coconut. It is colorless to pale brownish yellow. Coconut oil contains a high level of low molecular weight saturated fatty acids, lauric acid (Marina *et al.*, 2009b). The chemical composition of coconut oil makes it possible to use in a wide range of edible and non-edible purposes. Coconut oil has unique characteristics such as having bland flavor, pleasant odor, high resistance to rancidity, a narrow temperature range of melting, easy digestibility and absorbability, high gross for spray oil use and superior foam retention capacity for whip-topping use (Che Man and Marina, 2006; Marina *et al.*, 2009b).

Most commercial grade coconut oils can be produced from copra using smoke drying, sun drying or a combination of both. If standard copra is used as starting material, the unrefined coconut oil obtained is not suitable for consumption and must be purified. With unsanitary handling, drastic processing of copra for oil extraction and refining make the product susceptible to aflatoxin contamination and oxidative rancidity (Marina *et al.*, 2009b). The coconut oil must be further refined where the standard end product is RBD (Refined, Bleached and Deodorized) coconut oil. High heat is used in the deodorization of the oil and the oil is typically filtered through clays (bleaching) to removes impurities. Sodium hydroxide is generally used

to remove free fatty acids and prolong shelf life of the coconut oil. Both chemicals and high heat are usually used to improve the yield of oil from copra. RBD process is required to make the oil clear, tasteless and odorless. This process further removes the anti-oxidant and other components of the oil. The traditional way of producing refined coconut oil is through physical or mechanical refining (Guarte *et al.*, 1996).

1.2.6.1 Virgin coconut oil

Virgin Coconut Oil (VCO) is increasingly gaining wide popularity in the scientific field and among the public. It is getting global reputation as the healthiest and versatile oil and has gain a lot of attention in the world (Wong, 2010). Virgin coconut oil (VCO) is obtained from fresh and mature kernel (12 months old from pollination) of the coconut (*Cocos nucifera L.*) by mechanical or natural means with or without the application of heat, which does not lead to alteration of the nature of the oil. VCO has not undergone chemical refining, bleaching or deodorizing. It can be consumed in its natural state without the need for further processing (APCC, 2003; Codex, 2003). Virgin coconut oil consists mainly of medium chain fatty acids (MCFAs). MCFAs are burned up immediately after consumption and therefore the body uses it immediately to make energy, instead of storing it as body fat (Marina *et al.*, 2009b). Coconut oil contains 92% of saturated fatty acids (in the form of triglycerides), most of them (about 70%) are lower chain saturated fatty acids known MCFAs. It contains 45-56% MCFAs. Also, MCFAs are resistant to peroxidation. The fatty acids in virgin coconut oil are distinct from animal fats which contain mainly of long chain saturated fatty acids. Virgin coconut oil is colorless, free of sediment with natural fresh coconut scent. It is free from rancid odor or taste (APCC, 2003; Codex, 2003). VCO is rich in lauric acid, an essential fatty acid which transforms into a compound known as monolaurin acid that is believed to fight viral pathogens and protects the body from bacteria, viruses and infections from parasites (Wong, 2010). Besides lauric acid, VCO contains a considerable amount of short-chain fatty acids such as capric, caproic and caprylic acids which also show antimicrobial and antiviral properties (Marina *et al.*, 2009b). VCO has been claimed to have numerous beneficial health effects where it lowered total cholesterol, triglycerides, and phospholipids

(Marina *et al.*, 2009b). Butyric acid is used to treat cancer, while lauric acid is effective in treating viral infections (Marina *et al.*, 2009b).

Coconut oil obtained from copra, dried coconut, has no taste or fragrance, due to the refining process, whereas VCO has the natural fresh fragrance and taste of coconut which is free from rancid flavor and odors (Wong, 2010). The absence of heating and chemical treatment in the oil makes it tasty and healthy.

1.2.6.2 Manufacturing of virgin coconut oil

Based on the definition of virgin oil, it is understood that as long as the oil does not go through the RBD process and which does not lead to the alteration of the nature of the oil, the oil can be deemed as VCO (Marina *et al.*, 2009b). Coconut oil can be extracted using "dry" or "wet" processes

1.2.6.2.1 Dry extraction

Dry process requires the meat to be removed from the shell and dried using fire, sunlight, or kilns. The obtained copra is pressed or dissolved with solvents, producing the coconut oil with a high-protein and high-fiber mash. The mash is of poor quality for human consumption and is instead fed to ruminants. There is no process to extract protein from the mash. A portion of the oil extracted from copra is lost to some extent during extraction (Marina *et al.*, 2009b).

1.2.6.2.2 Wet extraction

Wet process or aqueous process is the term used for the extraction of coconut oil directly from coconut milk. The wet process uses raw coconut rather than dried copra, and the protein in the coconut creates an emulsion of oil and water (Grimwood *et al.*, 1975). The more problematic step is breaking up the emulsion to recover the oil. Coconut milk is a milky white oil-in-water emulsion and can be obtained by extraction from coconut flesh with or without added water (Raghavendra and Raghavarao, 2011). The emulsion in coconut milk was naturally stabilized by coconut proteins: globulins and albumins, and phospholipids (Raghavendra and Raghavarao, 2010). The oil can be separated from the emulsion by means of

enzymatic extraction, fermentation, refrigeration or mechanical centrifuge. Separation of the oil from emulsion can also be accomplished by breaking the emulsion (Wong, 2010). Wet extraction eliminates the use of solvent which reportedly may lower the investment cost and energy requirements. Furthermore, it eliminates the RBD process (Marina *et al.*, 2009b). Even though the concept appears potentially attractive, however, the method yields comparatively low content of oil, which has discouraged its commercial application (Rosenthal et al., 1996).

Destabilization of emulsion can be done through three mechanisms. The first stage is creaming by the action of gravitational force, resulting in two phases. The higher specific gravity phase takes place at the top and the lower specific gravity phase moves downward. The second stage is flocculation or clustering in which the oil phase moves as a group, which does not involve the rupture of the interfacial film that normally surrounds each globule and therefore does not change the original globule. For the last stage, coalescence is the most critical phase in destabilization. During this stage, the interfacial area is ruptured; the globules joined together and reduced the interfacial area (Wong, 2010). The wet process appears more desirable due to the free usage of chemical solvents, thus more environmental friendly than the solvent extraction (Marina *et al.*, 2009b). VCO can be separated by several wet extraction methods including enzymatic separation, physical separation, solvent separation, and fermentation (Marina *et al.*, 2009b).

1.2.6.2.2.1 Enzymatic separation

Recently, enzymatic pre-treatment emerging as a novel and an effective means to improve the oil yield (Marina *et al.*, 2009b). VCO can be separated from coconut milk by means of enzymatic hydrolysis (Raghavendra and Raghavarao, 2010). Separation of the oil from the water–oil emulsion can also be accomplished by breaking the emulsion using enzyme or aging for several hours, or by mechanical process using continuous centrifugation (Norulaini *et al.*, 2009). Among all processes, the enzyme-assisted separation process of coconut oil significantly increased the yield in aqueous system (Tano-Debrah and Ohta, 1997). Enzymatic separation is effective to release the oil by breaking down and destabilization the coconut emulsion (Rahayu

et al., 2008). VCO processed by enzymatic separation has more beneficial and safety effect than traditional method from copra, since they are often infected by insects or aflatoxin producing molds associated with the potential toxicity problem during manufacturing (Handayani *et al.*, 2009). The activity of enzyme is influenced by substrate and enzyme concentration, temperature, pH, and incubation time for enzymatic reaction (Handayani *et al.*, 2009). Those factors determined oil separation yield differently (Rahayu *et al.*, 2008). Raghavendra and Raghavarao (2010) reported that oil could be recovered from coconut milk hydrolyzed using papain with the yield of 60.09%. A maximum yield of 95.3 % was obtained when coconut milk was treated with aspartic protease at concentration of 0.02 mg/g (Raghavendra and Raghavarao, 2010).

In general, plant cell walls consist of complex carbohydrate molecules such as cellulose, hemicellulose, mannans, galactomanans, arabinogalactans, pectin substances and protein (Marina *et al.*, 2009b). Coconut meat contains about 10% of carbohydrate, in which 50% of this is cellulose and 75% of the cellulose is made up with cellulose (Marina *et al.*, 2009b). Oil is located inside plant cells, linked with proteins and wide range of carbohydrate such as starch, cellulose, hemicellulose, and pectins. To enhance the release of oil, cell-wall degrading enzymes can be used to extract oil by solubilizing the structural cell wall (Sant'Anna *et al.*, 2003). The maximal separation yield of oil was obtained when using 0.6% (w/w) Viscozyme L, followed by Neutrase 1.5 MG (0.3%, w/w), in which total incubation time of 60 min, temperature of 60°C, substrate/water ratio of 1:6 and pH of 7 were used (Sant'Anna *et al.*, 2003). Man *et al.* (1996) studied the use of an enzyme mixture at 1% (w/w) each of cellulase, α-amylase, polygalacturonase, and protease at pH 7.0 and an separation temperature of 60°C for coconut oil separation. The recovery of 73.8% and good-quality oil were achieved.

1.2.6.2.2.2 Physical separation

Chilling, freezing and thawing have been used to break the protein stabilized oil-in-water emulsion. Emulsion was centrifuged before chilling and thawing to allow better packing of the coconut oil globules (Seow and Gwee, 1997).

The temperature used for chilling and freezing were 10 °C and −4 °C, respectively while the thawing process was carried out at 40°C until the coconut cream reached room temperature (25°C). In addition, this action also helps in removing undissolved solids after separation process. The removal of solids present in high percentages in the dispersion of oil droplet was important for efficient recovery of oil by centrifugation (Rosenthal *et al.*, 1996). The centrifugation step was followed to enable the packing of cream oil globule to crystallize upon lowering the temperature. Centrifugation process was carried out at 2000-5000 g up to 6 min. During thawing, the oil coalesced and formed the large droplets of varying sizes. Raghavendra and Raghavarao (2010) reported that combination of treatments (enzyme treatment at 37 °C followed by chilling and thawing) of coconut milk emulsion resulted in the highest yield of 94.5%

1.2.6.2.2.3 Fermentation

Natural fermentation is a method where the less proceeding conditions were involved for production of VCO. In the natural fermentation process, coconut is allowed for microbial fermentation (Marina *et al.*, 2009b). In natural fermentation process of VCO production, normal flora of microorganisms will ferment the coconut milk and separates the coconut oil on the top portion within 24 - 48 h. During incubation, the fermentation associated with the microbial growth might enhance the destabilization of emulsion, plausibly mediated by microbial proteases. The separated oil can be collected. However, there was a chance of contamination with microorganisms because the coconut milk is the rich source of proteins, carbohydrates and moisture (Tansakul and Chaisawang, 2006) which can attract the microorganisms. Some microorganisms may spoil the coconut milk, resulting in poor quality VCO (generally in yellow color). Thus, the main disadvantages of this process are low oil recovery and fermented odor, which masks the characteristic coconut flavor of the oil (Raghavendra and Raghavarao, 2010). During fermentation of coconut milk, high FFA could be more produced by lipolytic enzymes in the presence of water. Coconut milk emulsion can also be separated by adjusting pH of the coconut milk emulsion between pH 3 and 5.6 and inoculated with bacteria cultures (Chen and Diosady, 2003). Man *et al.* (1997) investigated the use of pure culture, *Lactobacillus plantarum*

1041 IAM to separate coconut oil, which could be able to separate as much as 95% of the oil. VCO separation by induced fermentation under semi-controlled conditions using probiotic organisms (*Lactobacillus plantarum*) gives greater yields (28.47%) (Satheesh and Prasad, 2014).

1.2.6.3 Properties of virgin coconut oil

Different countries have different quality parameters for VCO. Those include Indian-Standard; IS: 6220-1971, Malaysian Standard and Philippines Standard, etc. Additionally, Codex (Codex, 2003) and the Asian and Pacific Coconut Community (APCC, 2003) have proposed international standard.

Table 7. Codex and Asian Pacific Coconut Community (APCC) Standards for Virgin Coconut Oil

Parameters	Codex Standards	APCC Standards
Relative density at 40°C/20°C	0.908-0.921	0.915 – 0.920
Refractive Index at 40°C	1.448-1.450	1.4480 – 1.4492
Moisture & other volatiles at 105°C	0.1	Max 0.1
Free fatty acids, calculated as lauric acid % by mass	Max 0.3	Max 0.2
Peroxide value (millequivalents of active oxygen per kg)	Not more than 15	Max 3
Iodine value	6.3-10.6	4.1 -11
Saponification Value	248-265	250 – 260
Unsaponifiables, % by mass, max g/kg	≤ 15	0.2-0.5
Polenske value	13 –18	13

1.3 Objectives

1.3.1 To purify and characterize protease from hepatopancreas of Pacific white shrimp.

1.3.2 To fractionate protease from hepatopancreas of Pacific white shrimp using phase partitioning systems.

1.3.3 To investigate autolysis profile and the impact of autolysis on lipid separation from the hepatopancreas of Pacific white shrimp.

1.3.4 To extract carotenoprotein from shrimp shells of Pacific white shrimp using proteases from Pacific white shrimp hepatopancreas.

1.3.5 To prepare gelatin hydrolysates from seabass skin with antioxidative activity using the proteases from Pacific white shrimp hepatopancreas.

1.3.6 To extract lipids from striped catfish muscle using the proteases from Pacific white shrimp hepatopancreas.

1.3.7 To extract virgin coconut oil from coconut milk using proteases from Pacific white shrimp hepatopancreas.

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CHAPTER 2
Purification and characterization of trypsin from hepatopacrease of Pacific white shrimp

2.1 Abstract

Trypsin from hepatopancreas of Pacific white shrimp (*Litopenaeus vannamei*) was purified to homogeneity using ammonium sulfate precipitation and a series of chromatographies including DEAE sepharose and SBTI-Sepharose 4B columns. Trypsin was purified to 50.4-fold with a yield of 13.7%. Based on native-PAGE, the purified trypsin showed a single band. Trypsin had a molecular weight of 24 kDa as estimated by SDS–PAGE. The optimal pH and temperature for α-*N*-benzoyl-dl-arginine-*p*-nitroanilide (BAPNA) hydrolysis were 8.0 and 60°C, respectively. Trypsin was stable to heat treatment up to 60°C, and over a pH range of 7.0–11.0. The activity was strongly inhibited by soybean *N*-*p*-tosyl-L-lysine chloromethyl ketone (TLCK). Purified trypsin had Michaelis–Menten constant (K_m) and catalytic constant (k_{cat}) of 1.60 mM and 3.33 s^{-1}, respectively, when BAPNA was used as the substrate. Trypsin with high k_{cat} indicated its high capacity of hydrolysis and it could serve as a promising protease.

2.2 Introduction

Shrimp hepatopancreas has been known as a potential source of different enzymes, especially proteases (Aoki *et al.*, 2003; Sriket *et al.*, 2012). The common proteases found in hepatopancreas of shrimp are trypsin, chymotrypsin and elastase (Aoki *et al.*, 2003; Gimenez *et al.*, 2001). Trypsin is able to cleave peptide chains, mainly at the carboxyl side of lysine or arginine, except when either is followed by proline (Kishimura *et al.*, 2008). Trypsin has been isolated and characterized from the hepatopancreas of various shrimp or prawn, including freshwater prawn (*Macrobrachium rosenbergii*) (Sriket *et al.*, 2012), white shrimp (*Penaeus setiferus*) (Gates and Travis, 1969), Indian prawn (*Penaeus indicus*) (Honjo *et al.*, 1990), Chinese white shrimp (*Penaeus orientalis*) (Oh *et al.*, 2000), crayfish (*Procambarus clarkii*) (Kim *et al.*, 1994), Northern shrimp (*Pandalus eous*) (Aoki *et*

al., 2003), Antarctic krill (*Euphausia superba*) (Wu *et al.*, 2014) and lobster (*Panulirus argus*) (Perera *et al.*, 2012).

Pacific white shrimp (*Litopenaeus vannamei*) is an economically important species in Thailand with high market value and has become the essential income generator of the country (Binsan *et al.*, 2008). By the year of 2012, frozen Pacific white shrimp and white shrimp products were manufactured and exported, mostly to USA and Japan, with total amount of 326,441 metric tons. During shrimp processing, a large amount of by-products including cephalothorax, shell, etc. is generated (Binsan *et al.*, 2008). Hepatopancreas free-whole shrimp is another product with increasing demand. During processing, hepatopancreas is removed by a vacuum sucking machine and can serve as the essential source of trypsin (Sriket *et al.*, 2012). Shellfish trypsin has been reported to be active in hydrolysis of proteinaceous substrates. Trypsin in hepatopancreas from freshwater prawn actively hydrolyzed collagens from freshwater prawn and yellowtail (Sriket *et al.*, 2012). Protease from Pacific white shrimp hepatopancreas could serve as an aid for a wide range of applications. However, no information regarding the properties and characteristics of trypsins from hepatopancreas of Pacific white shrimp exists. Therefore, this study aimed to purify and characterize trypsin from hepatopancreas of Pacific white shrimp.

2.3 Objective

To purify and characterize protease from hepatopancrease of Pacific white shrimp.

2.4 Material and methods

2.4.1 Chemicals

Soybean trypsin inhibitor (SBTI), α-*N*-benzoyl-dl-arginine-*p*-nitroanilide (BAPNA), *N*-*ρ*-tosyl-l-lysine-chloromethylketone (TLCK), ethylenediaminetetraacetic acid (EDTA), 1-(l-trans-epoxysuccinyleucylamino)-4-guanidinobutane (E-64), pepstatin A, *N*-tosyl-l-phenylalanine chloromethylketone (TPCK), phenylmethanesulfonyl fluoride (PMSF), *β*-mercaptoethanol (βME) and low molecular weight protein markers were purchased from Sigma Chemical Co. (St.

Louis, MO, USA). Coomassie brilliant blue G-250, sodium dodecyl sulfate (SDS) and *N*,*N*,*N*′,*N*″-tetramethyl ethylene diamine (TEMED) were obtained from Bio-Rad Laboratories (Hercules, CA, USA). Tris (hydroxymethyl) aminomethane (Tris–HCl) and ammonium sulphate ($(NH_4)_2SO_4$) were procured from Merck (Darmstadt, Germany). Diethylaminoethyl (DEAE) Sepharose™ Fast Flow and cyanobromide (CNBr)-activated Sepharose™ 4B were purchased from GE Healthcare Bio-sciences AB (Uppsala, Sweden).

2.4.2 Preparation of crude protease extract from hepatopancreas

Hepatopancreas of Pacific white shrimp was collected from Sea wealth frozen food Co., Ltd., Songkhla province, Thailand. Hepatopancreas was packaged in polyethylene bag, stored in ice using a sample/ice ratio of 1:2 (w/w) and transported to the Department of Food Technology, Prince of Songkla University within 2 h. Upon arrival, the samples were cut into pieces and ground into powder in the liquid nitrogen using a National Model MX-T2GN blender (Taipei, Taiwan). Prepared sample was homogenized in three volumes of acetone using a homogenizer (model PT-MR2100, POLYTRON®, KINEMATICA AG, Littau/Luzern, Switzerland) at 15,000 rpm at -20°C for 2 min according to the method of Kishimura and Hayashi (2002). The homogenate was stirred continuously using a magnetic stirrer model BIG SQUID (IKA®-Werke GmbH & CO.KG, Staufen, Germany) at -4°C for 30 min and filtered in vacuo on Whatman No. 4 filter paper (Whatman International Ltd., Maidstone, England). The residue obtained was then homogenized in two volumes of acetone (-20C) for 2 min and stirred at 4°C for 30 min. The homogenate was filtered as described above. Then the residue was allowed to stand at room temperature until dried and free of acetone odor. The obtained powder named ‘acetone powder’ was used for extraction of proteases.

To prepare the crude protease extract, acetone powder was suspended in an extraction buffer (10 mM Tris–HCl, pH 8.0 containing 1 mM $CaCl_2$) at a ratio of 1:50 (w/v) and stirred at 4°C for 3 h. The suspension was centrifuged for 10 min at 4°C at 10,000×g to remove the tissue debris using a refrigerated centrifuge (Beckman Coulter, Avanti J-E Centrifuge, Fullerton, CA, USA). The supernatant was filtered

through a Whatman filter paper No. 1. The filtrate obtained was referred to as “crude protease extract, CPE”.

2.4.3 Preparation of ammonium sulfate fraction

CPE was subjected to 40–60% saturated ammonium sulfate precipitation, according to the method of Khantaphant and Benjakul (2010) with a slight modification. From our preliminary study, the use of ammonium sulfate at 40-60% saturation showed the highest yield and purity, in comparison with ammonium sulfate with other ranges (0-20%, 20-40%, 60-80%). After the addition of ammonium sulfate, the mixture was stirred gradually at 4°C for 30 min. Thereafter, the mixture was centrifuged at 8000×g for 30 min at 4°C and the pellet obtained was dissolved in the minimum volume of the extraction buffer. The solution was dialyzed against 20 volumes of the extraction buffer overnight at 4°C with three changes of dialysis buffer. The dialysate was kept in ice and referred to as “ammonium sulfate fraction, ASF”

2.4.4 Purification of trypsin

ASF was subjected to a series of chromatographies, including ion-exchange column and affinity column. All purification steps were conducted at room temperature (25°C). Firstly, ASF was applied onto a DEAE-Sepharose column (1×30 cm) pre-equilibrated with 10 mM Tris–HCl, pH 8.0, containing 1 mM $CaCl_2$. The column was washed with the same buffer until the absorbance of 280 nm was less than 0.005. The elution was then performed using a linear gradient of NaCl from 0 to 1.25 M in 10 mM Tris–HCl, pH 8.0, containing 1 mM $CaCl_2$ with a flow rate of 0.5 mL/min. For all fractions, the protein content was monitored using OD_{280}. After separation using the column, all fractions (2 mL) were collected, kept at 4°C until testing for trypsin activity. Active fractions were pooled and dialyzed against 10 volumes of 50 mM Tris–HCl buffer, pH 8.0, containing 10 mM $CaCl_2$ with two changes of dialysis buffer at 4°C. The dialysate was further subjected to soybean trypsin inhibitor (SBTI)-Sepharose4B column.

SBTI-Sepharose 4B column was prepared following the instructions of the supplier. CNBr-activated Sepharose 4B was swollen and washed in 200 volumes of 1 mM HCl. The coupling between Sepharose gel and SBTI (1:0.027 (w/w)) was carried out at room temperature and the remaining active group on the medium was blocked by 0.1 M Tris–HCl, pH 8.0. Thereafter, SBTI-Sepharose 4B column (1 × 10 cm) was pre-equilibrated with 50 mM Tris–HCl, pH 8, containing 10 mM $CaCl_2$ and 0.5 M NaCl. DEAE-Sepharose fraction was then loaded into the column. The column was washed with the same buffer containing no $CaCl_2$ with a flow rate of 0.5 mL/min until the absorbance of 280 nm was less than 0.005. The elution was carried out using 5 mM HCl with a flow rate of 1.0 mL/min. Fractions (2 ml) were collected and each fraction was mixed rapidly with 0.5 mL of 100 mM Tris–HCl, pH 8.5, containing 10 mM $CaCl_2$. OD_{280} of fractions was determined. The fractions were kept at 4°C until assayed for trypsin activity. Active fractions were pooled and dialyzed against 10 volumes of 10 mM Tris–HCl, pH 8.0, containing 1 mM $CaCl_2$ at 4°C. Two changes of dialysis buffer were performed during dialysis. The dialysate referred to as 'SBTI-Sepharose 4B fraction' was lyophilized and stored at –40°C.

2.4.5 Assay for trypsin activity

Trypsin activity was measured using BAPNA as a substrate according to the method of Khantaphant and Benjakul (2010). A 200 µL of sample was mixed with 200 µL of distilled water and 1000 µL of reaction buffer (50 mM Tris–HCl buffer, pH 8.0, containing 10 mM $CaCl_2$). The reaction was initiated by adding 200 µL of 2 mg/mL BAPNA to the reaction mixture. After incubation for 15 min at 60°C, 200 µL of 30% acetic acid (v/v) were added to terminate the reaction. Production of *p*-nitroaniline was measured by monitoring the absorbance of reaction mixture at 410 nm (A_{410}) using a UV-1601 spectrophotometer (Shimadzu, Kyoto, Japan). A blank was conducted in the same manner except that the sample was added after addition of 30% acetic acid. One unit was defined as the amount of trypsin causing an increase of 1.0 in A_{410} per min.

2.4.6 SDS–polyacrylamide gel electrophoresis (SDS–PAGE) and native-PAGE

SDS–PAGE was performed according to the method of Laemmli (1970). Enzyme solutions were mixed at 1:1 (v/v) ratio with the SDS–PAGE sample buffer (0.125 M Tris–HCl, pH 6.8; 4% SDS; 20% glycerol; 10% β-mercaptoethanol) and boiled for 3 min. The samples (10 μg) were loaded onto the gel made of 4% stacking and 15% separating gels and subjected to electrophoresis at a constant current of 15 mA per gel using a Mini-Protean II Cell apparatus (Atto Co., Tokyo, Japan). After electrophoresis, the gels were stained with 0.1% Coomassie brilliant blue R-250 in 50% methanol and 7% acetic acid and destained with 7% acetic acid.

Native-PAGE was performed using 15% separating gels in a similar manner, except that the sample was not heated and SDS and reducing agent were omitted from sample buffer and the system.

2.4.7 Substrate-gel electrophoresis (Activity staining)

Enzyme extracts were separated on SDS-PAGE, followed by activity staining according to the method of Sriket *et al.* (2012). Enzyme solutions were mixed with sample buffer at a ratio of 1:1 (v/v) without heating. Two μg of proteins were loaded into the gel made of 4% stacking and 15% separating gels. The proteins were subjected to electrophoresis as previously described. After electrophoresis, the gels were immersed in 100 mL of 50 mM Tris–HCl buffer, pH 7.5, containing 2% casein (w/v) for 1 h at 0°C with a gentle agitation. Thereafter, the gels were transferred to 2% casein (w/v) in 50 mM Tris–HCl buffer (pH8). The mixture was incubated at 60°C for 15 min with continuous agitation. The gels were then stained with 0.05% Coomassie brilliant blue R-250 in 15% methanol and 5% acetic acid and destained in 30% methanol and 10% acetic acid. Protease band was indicated by the development of a clear zone on a blue background.

2.4.8 pH and temperature profile

Trypsin activity was assayed over the pH range of 2.0–11.0 (50 mM acetate buffer for pHs 2.0–7.0; 50 mM Tris–HCl buffer for pHs 8.0–9.0 and 50 mM glycine–NaOH for pHs 9.0–11.0) at 60°C for 10 min. For the temperature profile study, the activity was assayed at different temperatures (20, 30, 40, 50, 55, 60, 65, 70 and 80°C) for 10 min at pH 8.0.

2.4.9 pH and thermal stability

The effect of pH on stability of trypsin was evaluated by measuring the residual activity after incubation at various pHs. Enzyme solution was mixed with different buffers mentioned above at a ratio of 1:1 (v/v) for 30 min at 30°C. Thereafter, the residual activity was assayed at 60°C and pH 8 using BAPNA as substrate. For thermal stability, the enzyme solution was mixed with 100 mM Tris–HCl, pH 8.0 at a ratio of 1:1 (v/v) and incubated at different temperatures (20, 30, 40, 50, 55, 60, 65, 70 and 80°C) for 15 min in a temperature controlled water bath (model W350, Memmert, Schwabach, Germany). The treated samples were suddenly cooled in iced water. The residual activity was assayed using BAPNA as a substrate at pH 8.0 and 60°C.

2.4.10 Effect of inhibitors

The effect of inhibitors on trypsin activity was determined according to the method of Sriket *et al.* (2012). Enzyme solution was mixed with an equal volume of protease inhibitor solution to obtain the final concentration designated (0.1 mM E-64, 1.0 g/L soybean trypsin inhibitor, 5 mM PMSF, 5 mM TLCK, 5 mM TPCK, 1 mM pepstatin A and 10 mM EDTA). The mixture was allowed to stand at room temperature (25°C) for 15 min. Thereafter, the remaining activity was measured and percentage inhibition was calculated.

2.4.11 Kinetic studies

Kinetic studies of purified trypsin were carried out according to the method of Khantaphant and Benjakul (2010) using BAPNA with concentrations , ranging from 0.01 to 10.0 mM, as the substrate. The final enzyme concentration for

the assay was 0.01 mg protein/mL. The kinetic parameters, including the maximal velocity (V_{max}) and Michaelis–Menten constant (K_m), were evaluated at pH 8.0 and 30°C, using a Lineweaver–Burk double-reciprocal plot (Lineweaver and Burk 1934). Catalytic constant (k_{cat}) was calculated from the following equation: $k_{cat} = V_{max}/[E]$, where [E] is molar concentration of enzyme calculated, based on the molecular weight determined by SDS–PAGE and protein concentration.

2.4.12 Protein determination

Protein concentration was measured by the Bradford method using bovine serum albumin (BSA) as a protein standard (Bradford, 1976).

2.4.13 Statistical analysis

A completely randomized design was used throughout this study. Experiments were run in triplicate. Data was expressed as mean ± SD. Data were subjected to analysis of variance (ANOVA). Comparison of means was carried out by Duncan's multiple range tests (Steel and Torrie, 1980). Statistical analysis was performed using the Statistical Package for Social Science (SPSS 11.0 for windows, SPSS Inc., Chicago, IL, USA).

2.5 Results and dicussion

2.5.1 Purification of trypsin from hepatopancreas of Pacific white shrimp

Table 8 Purification of trypsin from hepatopancreas of Pacific white shrimp

Purification steps	Total protein (mg)	Total activity (unit)[a]	Specific activity (unit/mg)	Yield (%)	Purity (fold)
Crude protease extract	1687.0	2250463	1334	100.0	1.0
$(NH_4)_2SO_4$ (40–60%)	536.8	1364321	2542	60.6	1.9
DEAE Sepharose	32.5	790590	24326	35.1	18.2
SBTI-Sepharose 4B	4.6	309000	67174	13.7	50.4

[a] Trypsin activity was assayed at pH 8.0 and 60°C for 15 min using BAPNA as a substrate.

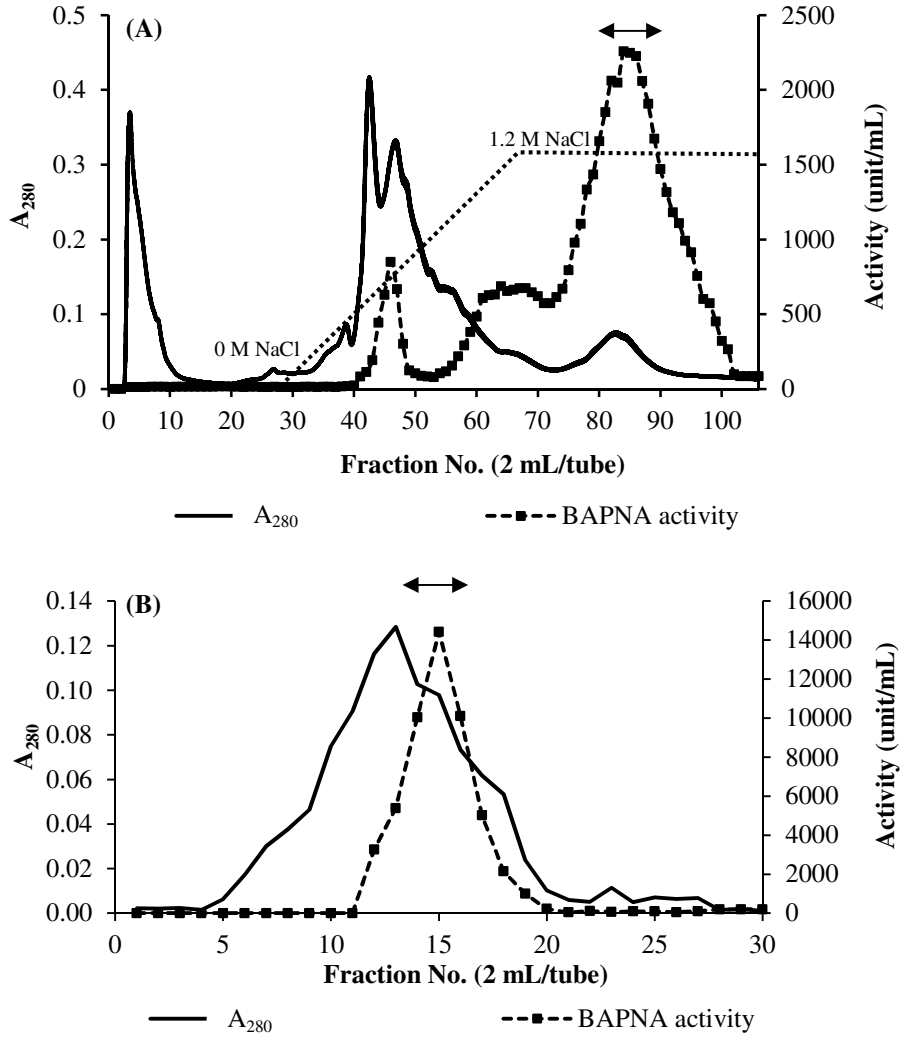

Figure 2 Elution profiles of ammonium sulfate (40–60% saturation) fraction on DEAE-sepharose column (A) and SBTI-Sepharose 4B column (B).Elution was performed using a gradient (0-1.25 M NaCl) with a flow rate of 0.5 mL/minfor DEAE Sepharose column and 5 mMHCl with a flow rate of 1.0 mL/min for the SBTI-Sepharose 4B column. Fractions (2 mL) were determined for trypsin activity using BAPNA as a substrate. (↔): pooled fractions.

Trypsin was purified from the hepatopancreas of Pacific white shrimp by a series of chromatographies after precipitation with ammonium sulfate (40–60% saturation) as summarized in Table 8. After being precipitated by ammonium sulfate, the obtained fractions had the increase in purity by 1.9-fold. Ammonium sulfate precipitation is a simple method and is generally introduced as an initial step to

remove other proteins from the crude extract (Khantaphant and Benjakul, 2010). When ammonium sulfate fraction was subjected to DEAE Sepharose column, two activity peaks were obtained (Figure 2A). Purity of 18.2-fold were observed after being chromatographed using this anion exchange column. Ion-exchange chromatography was used to remove contaminating proteins and to separate different trypsin isoforms (Khantaphant and Benjakul, 2010). The pooled fractions with trypsin activity were then subjected to SBTI-Sepharose 4B column (Figure 2B). This purification step resulted in an increase in purity by 50.4-fold, compared with that of CPE, and a yield of 13.7% was obtained. SBTI-Sepharose 4B column, an affinity column, has been used to purified trypsin from pyloric caeca of bigeye snapper (Van Hau and Benjakul, 2006) and brown stripe red snapper (Khantaphant and Benjakul, 2010). SBTI can bind with trypsin specifically, while other contaminating proteins and enzymes can be re moved via washing (Khantaphant and Benjakul, 2010).

2.5.2 Purity and molecular weight

Based on SDS-PAGE, CPE contained several protein bands, representing sarcoplasmic proteins as well as enzymes e.g. proteases. After being purified using SBTI-Sepharose 4B column, only one single band was observed (Figure 3A). The results indicated that DEAE sepharose anion exchange and SBTI-Sepharose 4B column chromatographies effectively removed the contaminants from the crude extract. As a consequence, the purified trypsin was obtained. Molecular weight of trypsin was estimated to be 24 kDa. Trypsin is a serine protease, which consists of a single peptide chain with MW typically 24 kDa (Torrissen and Male, 2000). The activity band was confirmed by substrate gel electrophoresis, in which a clear band with the same molecular weight appeared on a blue background (Figure 3B).

From native gel-electrophoresis, only a single protein band was obtained (Figure 3C), confirming that trypsin was purified to homogeneity. It was noted that the activity bands were observed in CPE with MW of 48, 27 and 24 kDa. Nevertheless, only band with MW of 24 kDa was found in SBTI-Sepharose 4B fraction. This suggested the removal of protease with MW of 48 kDa and 27 kDa after purification using DEAE Sepharose column and SBTI-Sepharose 4B column.

Trypsins have been isolated and characterized from hepatopancreas or intestines of fish and shellfish. Trypsin from the hepatopancreas of freshwater prawn with MW 17 kDa was purified using a series of chromatographies including Q-Sepharose, Superdex 75 and MonoQ columns (Sriket *et al.*, 2012). Trypsin from hepatopancreas of shrimp *Penaus indicus* had MW of 36 kDa (Honjo *et al.*, 1990), whilst, trypsin from digestive gland of the shrimp *Penaeus japonicus* had MW of 25 kDa (Galgani *et al.*, 1985). Trypsin in hepatopancreas of lobster (*Panulirus argus*) had MW of 35-36 kDa (Perera *et al.*, 2012). Trypsin-like enzymes from various crustaceans vary in MW (Sriket *et al.*, 2012).

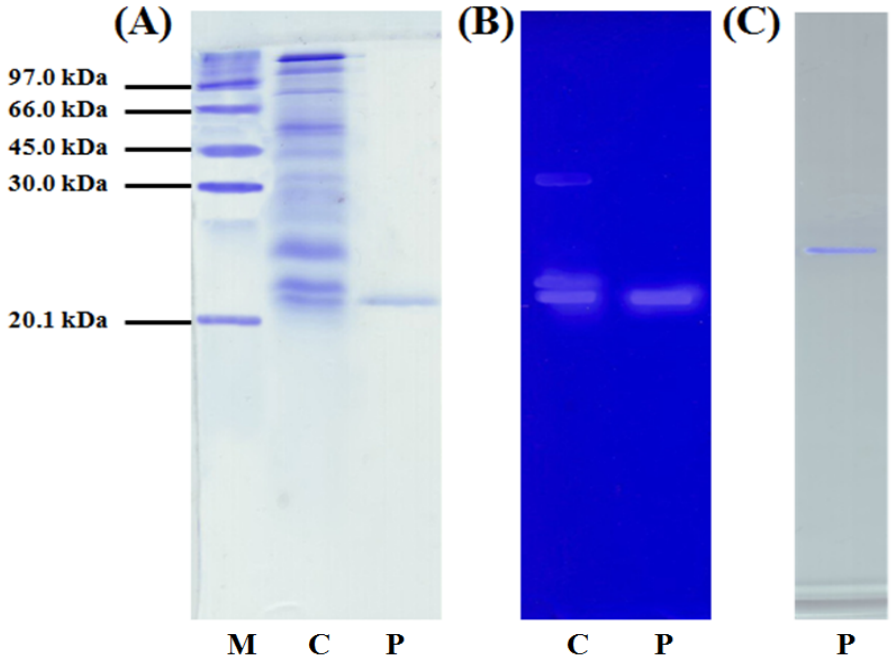

Figure 3 SDS–PAGE (A), activity staining (B) and native-PAGE (C) of purified trypsin from hepatopancreas of Pacific white shrimp. M, molecular weight standard; C, Crude protease extract; P, purified trypsin.

2.5.3 pH and temperature profiles

Temperature and pH profiles of purified trypsin from hepatopancreas of Pacific white shrimp are shown in Figure 4A and 4B, respectively. The optimal pH for BAPNA hydrolysis was 8. The optimum pHs of trysin from hepatopancreas of lobster (*Panulirus argus*) were pH 7-8, using BAPNA as a substrate (Perera *et al.*,

2012). The lowered activity at very acidic and alkaline pH was plausibly due to the conformational changes of enzyme under harsh conditions (Sriket *et al.*, 2012; Vega-Villasante *et al.*, 1995). Trypsin from Antarctic krill also showed the maximal activity using BAPNA as substrate at pH 8 (Bustos *et al.*, 1999). The optimal temperature of trypsin was 60°C ($p<0.05$), when BAPNA was used as a substrate. However, the activity markedly decreased at temperatures above 60°C, mainly due to thermal denaturation of trypsin (Khantaphant and Benjakul, 2010; Sriket *et al.*, 2012). The same optimal temperature (60°C) was reported for trypsin from shrimp *Penaeus japonicus* (Galgani *et al.*, 1985), shrimp *Penaus indicus* (Honjo *et al.*, 1990), and Antarctic krill (Bustos *et al.*, 1999) when BAPNA was used as a substrate. Nevertheless, trypsin from hepatopancreas of Pacific white shrimp had higher optimal temperature than those from some fish and shellfish, which had optimal temperatures in the range of 40–45°C such as 45°C for shrimp *Penaus indicus* (Honjo *et al.*, 1990), 40-50°C for North Pacific krill (Wu *et al.*, 2014), 50°C for sardinella (Ben Khaled *et al.*, 2008) and 60°C for lobster (*Panulirus argus*) (Perera *et al.*, 2015).

2.5.4 pH and temperature stability

The pH stability of trypsin from hepatopancreas of Pacific white shrimp is shown in Figure 4C. Trypsin was stable in pHs ranging from 7.0 to 11.0. The loss in activity was more pronounced at pH values below 7. A complete loss in activity was observed after exposure to pH 2. The results indicated the irreversible denaturation of trypsin at acidic pHs (Khantaphant and Benjakul, 2010). However, a slight decrease in activity was found at pH above 9. Trypsins from freshwater prawn (Sriket *et al.*, 2012), crawfish (Jeong *et al.*, 2000) North Pacific krill (Wu *et al.*, 2014) and shrimp *Penaeus japonicas* (Galgani *et al.*, 1985) were stable in the neutral and alkaline pH ranges, in which 80–100% of activity was retained. Crustacean trypsin is unstable at acidic pH, and contains a lower content of basic amino acid residues in the polypeptide chain in comparison with mammalian trypsins (Gates and Travis, 1969). Inactivation at acidic pH is a common phenomenon for anionic trypsins (Kim *et al.*, 1994).

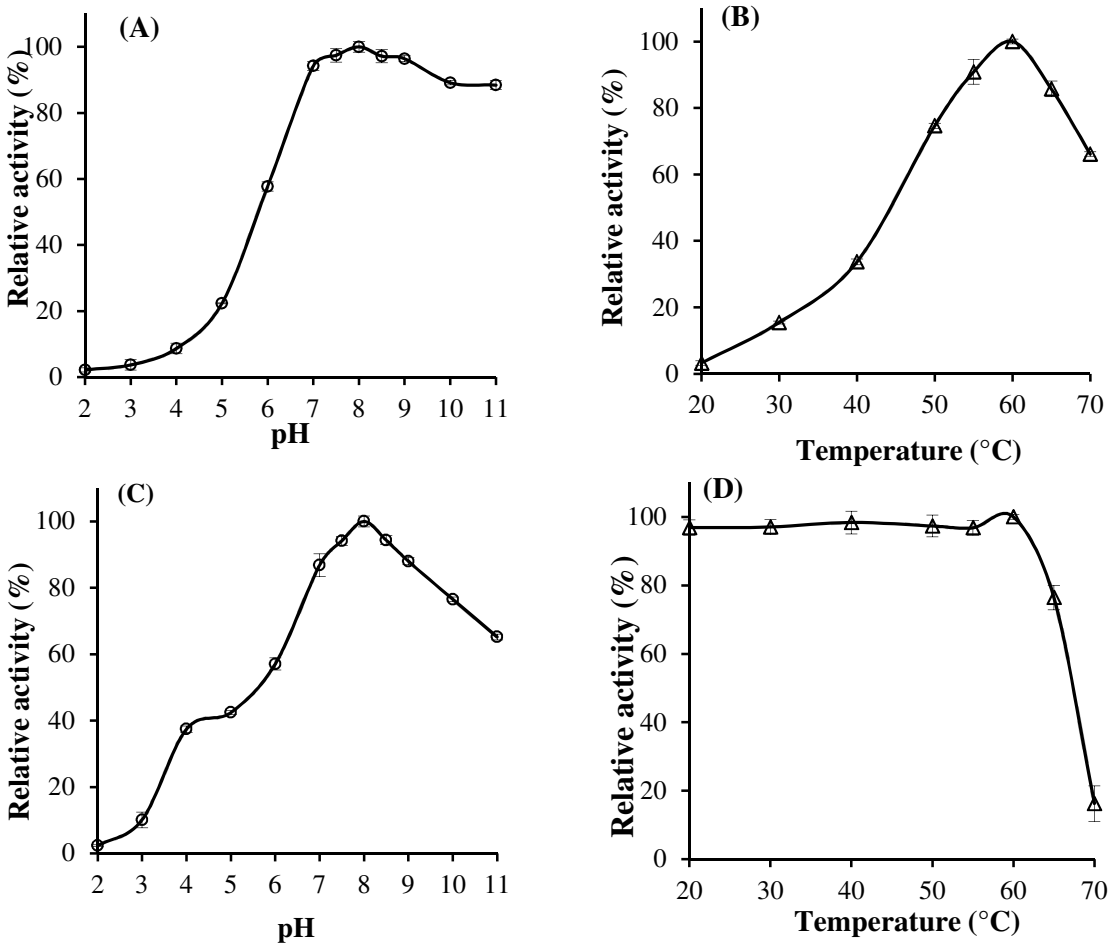

Figure 4 pH and temperature profiles (A and B) and pH and thermal stabilities (C and D) of purified trypsin from hepatopancreas of Pacific white shrimp. Bars represent the standard deviation (*n=3*).

Thermal stability of purified trypsin from hepatopancreas of Pacific white shrimp is depicted in Figure 4D. Trypsin was quite stable up to 60°C with a residual activity of 95–99%. Decreases in activity were observed when heated at temperatures above 60°C. At high temperatures, trypsin most likely underwent denaturation and lost its activity. Sriket *et al.* (2012) found that trypsin activity from hepatopancreas of freshwater prawn was stable up to 40°C and the activity disappeared when it was heated at temperature above 80°C. Trypsin from the hepatopancreas of shrimp *Penaeus omentaim* was stable within the temperature range of 40-50°C for 15 min, but lost its activity rapidly at temperatures above 50°C (Oh *et*

al., 2000). High thermal stability of trypsin from Pacific white shrimp hepatopancreas could be beneficial for its applications at high temperature.

2.5.5 Effect of inhibitors

The effect of various inhibitors on the activity of trypsin from hepatopancreas of Pacific white shrimp was determined as shown in Table 9. Trypsin was markedly inhibited by serine protease inhibitors, including soybean trypsin inhibitor, TLCK and PMSF. Amongst those protease inhibitors, TLCK showed the highest inhibitor (99.8 %) (p<0.05). Soybean trypsin inhibitor forms a stable stoichiometric enzymatically inactive complex with trypsin, thereby reducing the availability of trypsin (Valdez-Melchor *et al.*, 2013), whilst TLCK is a competitive inhibitor with trypsin (Kim and Jeong, 2013). When the enzyme binds to TLCK at active site, the substrate cannot be hydrolyzed. PMSF binds specifically to the active site serine residue in a serine protease (Barkia *et al.*, 2010). It affords alkenes by reacting with carbonyl compounds and causes sulfonylation of the active-site serine residues (Barkia *et al.*, 2010). Sriket *et al.* (2012) reported that the activities of trypsin from hepatopancreas of freshwater prawn were inhibited by soybean trypsin inhibitors and TLCK. The enzymatic activities of two collagenolytic proteases from the hepatopancreas of Northern shrimp were also effectively inhibited by PMSF (Aoki *et al.*, 2003). TPCK, a competitive inhibitor of chymotrypsin (Sriket *et al.*, 2012), showed very low inhibition toward trypsin. EDTA also exhibited a low inhibitory activity towards trypsin. EDTA possibly chelated Ca^{2+}, which was known to activate trypsin to some extent (Kishimura and Hayashi, 2002). Cysteine and aspartic protease inhibitors involving E-64 and pepstatin A, respectively, did not show inhibitory effects towards trypsin. The result confirmed that the purified enzyme was serine proteases, most likely trypsin.

Table 9 Effect of various inhibitors on the activity of purified trypsin from hepatopancreas of Pacific white shrimp[a].

Inhibitors	Concentration	% Inhibition
Control	–	0
Pestain A	1 mM	0
E-64	0.1 mM	0
SBTI	1 g/L	$98.3 \pm 0.1^{b*}$
TLCK	5 mM	99.8 ± 0.1^{a}
TPCK	5 mM	10.3 ± 1.6^{d}
PMSF	5 mM	96.8 ± 0.8^{c}
EDTA	10 mM	9.3 ± 1.2^{d}

[a] Enzyme solution was incubated with the same volume of inhibitor at 25°C for 15 min and the residual activity was determined using BAPNA as substrate for 15 min at pH 8 and 60°C.

* Means ± the standard deviation (n = 3).

Different superscripts indicate the significant differences (p<0.05)

2.5.6 Kinetic study

Kinetic parameters for purified trypsin from hepatopancreas of Pacific white shrimp are summarized in Table 10. K_m and k_{cat} for the hydrolysis of BAPNA were 0.40 mM and 3.33 s^{-1}, respectively. The catalytic efficiency (k_{cat}/K_m) for the hydrolysis of BAPNA was calculated to be 8.33 $s^{-1}mM^{-1}$. For the BAPNA hydrolysis, trypsin from hepatopancreas of Pacific white shrimp had a higher K_m value, than those from brownstripe red snapper (Khantaphant and Benjakul, 2010), cuttlefish (Balti *et al.*, 2009), bigeye snapper (Van Hau and Benjakul, 2006) and Monterey sardine (Castillo-Yánez *et al.*, 2005). K_m is often associated with the affinity of the enzyme for substrate (Khantaphant and Benjakul, 2010; Villalba-Villalba *et al.*, 2013). Therefore, trypsin from hepatopancreas of Pacific white shrimp had a higher affinity for BAPNA than brown stripe red snapper.

Trypsin from hepatopancreas of Pacific white shrimp showed higher k_{cat} than did those from cuttlefish (Balti *et al.*, 2009), bigeye snapper (Van Hau and Benjakul, 2006) and Monterey sardine (Castillo-Yánez *et al.*, 2005). k_{cat} indicates the maximum number of enzymatic reactions catalyzed per second(Whitaker and Bender,

1965). Thus, the higher k_{cat} of trypsin from hepatopancreas suggested a higher rate of substrate hydrolysis, in comparison with other trypsins.

Catalytic efficiency (k_{cat}/K_m) of trypsin from hepatopancreas of Pacific white shrimp was lower than those from brown stripe red snapper (Khantaphant and Benjakul, 2010), cuttlefish (Balti *et al.*, 2009) and Monterey sardine (Castillo-Yáñez *et al.* 2005) when BAPNA was used as a substrate. However, the purified trypsin showed a higher catalytic efficiency than did trypsin from bigeye snapper (Van Hau and Benjakul, 2006) when BAPNA was used as a substrate. The higher catalytic efficiency, the more efficient the enzyme is in transforming the substrate to product (Whitaker and Bender, 1965). This result suggested that trypsin from hepatopancreas of Pacific white shrimp was more effective in hydrolysis of BAPNA than trypsin from bigeye snapper.

Table 10 Kinetic parameters of purified trypsin from hepatopancreas of Pacific white shrimp trypsin and other fish trypsins.

Trypsins	K_m * **(mM)**	k_{cat} * **(s^{-1})**	k_{cat}/K_m **($s^{-1}mM^{-1}$)**	**References**
Pacific white shrimp (*Litopenaeus vannamei*)**	0.40	3.33	8.33	This study
Brownstripe red snapper (*Lutjanus vitta*)**	0.51	4.71	9.27	Khantaphant and Benjakul (2010)
Cuttlefish (*Sepia officinalis*)**	0.064	2.32	36.25	Balti *et al*. (2009)
Bigeye snapper (*Priacanthus macracanthus*)**	0.31	1.06	3.4	Van Hau and Benjakul (2006)
Montery sardine (*S. sagax cearula.*)**	0.051	2.12	41.0	Castillo-Yanez *et al*. (2005)

* K_m and k_{cat} values of all trypsins were determined at 30°C under the optimal pH.
** Substrate: *N*α -benzoyl-DL-arginine-*p*-nitroanilide (BAPNA).

2.6 Conclusion

The purified protease from hepatopancreas of Pacific white shrimp was classified as trypsin, based on the ability to hydrolyze specific synthetic substrates, molecular weight, optimal conditions of reaction and the response to a specific trypsin inhibitor. Trypsin exhibited the highest hydrolytic activity toward BAPNA at 60°C and pH 8.0. Therefore, hepatopancreas of Pacific white shrimp could be an important source of trypsin for further uses.

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CHAPTER 3

Use of the combined phase partitioning systems for recovery of proteases from hepatopancreas of Pacific white shrimp

3.1 Abstract

Recovery of proteases from hepatopancreas of Pacific white shrimp using three-phase partitioning (TPP) system in combination with aqueous two-phase system (ATPS) was investigated. TPP was performed using *t*-butanol with different crude protease extract (CPE)/*t*-butanol ratios. The interphase of system comprising CPE and *t*-butanol with a ratio of 1:1 in the presence of 30% ammonium sulfate yielded the highest purification fold (PF) (2.64-fold) with the recovery of 76.0%. Subsequently, TPP fraction was subjected to ATPS. Effects of phase compositions including PEG molecular weight (MW) and concentration as well as types and concentration of salts on partitioning of proteases were studied. ATPS comprising PEG1000 (15%, w/w) and magnesium sulfate (25%, w/w) provided the best condition for the maximal partitioning of proteases into the top phase and gave the highest PF (8.59-fold). The yield of 65.5% was obtained. ATPS fraction was further mixed with PEG8000 and several salts for back extraction (BE). BE using 25% PEG8000 and 10% MgSO4 gave the highest PF (9.94-fold) with the yield of 46.16%. Based on electrophoresis and activity staining, the fractionated proteases had the MW of 36 and 26 kDa. Therefore, the combined partitioning systems, TPP-ATPS-BE, could be effectively used to recover and purify proteases from hepatopancreas of Pacific white shrimp.

3.2 Introduction

Pacific white shrimp and its products have become economically important for Thailand. By the year 2010, frozen Pacific white shrimp and products were manufactured and exported totally for 407,978 metric tons, particularly to USA and Japan (Lebel *et al.*, 2010). During shrimp processing, a large amount of by-products including cephalothorax, shell, etc. is generated (Binsan *et al.*, 2008). Whole

shrimp without hepatopancreas is another product with increasing demand. Practically, hepatopancreas is removed by vacuum sucking machine and can serve as the potential source of protease (Binsan *et al.*, 2008; Sriket *et al.*, 2012). Fish proteases e.g. trypsin and cymotrypsin may have some unique properties for industrial applications, e.g. in the detergent, food, pharmaceutical, leather and silk industries (Klomklao *et al.*, 2005; Nalinanon *et al.*, 2009). Serine protease especially, trypsins, have been reported as the dominant proteases in hepatopancreas of freshwater prawn (Sriket *et al.*, 2012), kuruma prawn (*Penaeus japonicus*) (Galgani *et al.*, 1985), Indian prawn (Honjo *et al.*, 1990), Northern shrimps (*Pandalus borealis*) and white shrimp (*Penaeus vannamei*) (Ezquerra *et al.*, 1997).

TPP is a bioseparation technique which may be used for fractionation of enzymes and proteins from aqueous solutions (Rawdkuen *et al.*, 2010). $(NH_4)_2SO_4$ with certain saturation is generally added to precipitate the protein, and *t*-butanol is added to make three-phase layers (Chaiwut *et al.*, 2010). In general, biomolecules are recovered in a purified form at the interphase, while the contaminants such as lipids mostly partition in *t*-butanol (top phase) and aqueous phase (bottom phase) (Rawdkuen *et al.*, 2012).

ATPS has been used successfully for the separation and purification of proteins or enzymes (Klomklao *et al.*, 2005). ATPS forms readily upon mixing aqueous solutions of two hydrophilic polymers, or of a polymer and a salt, above a certain threshold. Phase separation occurs over certain concentrations of phase component concentration (Nalinanon *et al.*, 2009). ATPS has been applied for partitioning and recovery of various proteases such as pepsin (Nalinanon *et al.*, 2009), chymosin (Reh *et al.*, 2007), etc. In general, ATPS yields a specific environment suitable for maintaining enzymes in their native structure and for selectively partitioning of the enzyme to one of the phases (Nalinanon *et al.*, 2009). Coincidentally, ATPS can remove undesirable enzymes/proteins, unidentified polysaccharides and pigments that are present in the system (Babu *et al.*, 2008). These systems have good resolution, low material cost, high yield, less energy consumption, and less process time. In general, the single system has been implemented for enzyme recovery. The use of combined partitioning systems, e.g. TTP and ATPS could be an advantageous separation method to increase the purity of target enzyme as well as to

increase the recovery yield. Our objective was to investigate the use of combined TPP and ATPS for partitioning and recovery of proteases from hepatopancreas of Pacific white shrimp.

3.3 Objective

To fractionate protease from hepatopancrease of Pacific white shrimp using partitioning systems.

3.4 Materials and methods

3.4.1 Chemicals

Polyethylene glycol (PEG) 1000, PEG2000, PEG4000, PEG8000, *tert*-butanol (*t*-butanol) and tris (hydroxymethyl) aminomethane were obtained from Merck (Darmstadt, Germany). Benzoyl-*DL*-arginine-*p*-nitroanilide (BAPNA), sodium caseinate, bovine serum albumin (BSA), wide range molecular weight markers and Coomassie Brilliant Blue G-250 were purchased from Sigma Chemical Co. (St. Louis, MO, USA). Sodium dodecyl sulphate (SDS) was obtained from Fluka (Buchs, itzerland). *N*,*N*,*N*′,*N*′-tetramethyl ethylene diamine (TEMED) was purchased from Bio-Rad Laboratories (Hercules, CA, USA). The salts and other chemicals with the analytical grade were procured from Merck (Darmstadt, Germany).

3.4.2 Preparation of crude protease extract

Hepatopancreas of Pacific white shrimp was powdered in liquid nitrogen using a blender (Philips, Guangzhou, China) until the fine powder was obtained. The powder was then homogenized using a homogenizer (IKA Labortechnik, Selangor, Malaysia) in three volumes of acetone (-20°C) for 2 min, followed by stirring for 30 min according to the method of Kishimura and Hayashi (2002). The homogenate was filtered in vacuo on Whatman No. 4 filter paper (Whatman International Ltd., Maidstone, England). The residue obtained was then stirred in two volumes of acetone (-20°C) for 30 min. The residue was left at room temperature until dried and free of acetone odor.

To prepare the crude extract, acetone powder was suspended in 10 mM Tris–HCl, pH 8.0 containing 1 mM $CaCl_2$ at a ratio of 1:50 (w/v) and stirred continuously at 4°C for 3 h. The suspension was centrifuged using a refrigerated centrifuge (Beckman Coulter, Avanti J-E Centrifuge, Fullerton, CA, USA) for 10 min at 4°C at 10,000×*g* to remove the tissue debris, and the supernatant was referred to as "crude protease extract, CPE".

3.4.3 Three-phase partitioning (TPP) system

TPP was carried out as described by CPE with protein content of 5.97 mg/mL was used. Firstly, CPE was added with *t*-butanol at different ratios (1.0:0.5, 1.0:1.0, 1.0:1.5, and 1.0:2.0 (v/v)). Thereafter, ammonium sulfate at 30% saturation was added into the mixture. The mixtures were mixed thoroughly and then allowed to stand for 60 min at 4°C before subjecting to centrifugation at 5000×*g* for 10 min to facilitate the separation of phases. The lower aqueous layer and the interfacial phase were collected and dialyzed against 50 volumes of distilled water overnight at 4°C with 4 changes of distilled water. After dialysis, the samples were determined for protease activity and total protein content. Yield, specific activity (SA) and purification fold (PF) were calculated as follows:

$$\text{Yield (\%)} = \frac{A_t}{A_i} \times 100$$

where A_t is total protease activity in the protease rich phase and A_i is the initial protease activity of the crude extract or fraction before being partitioned.

$$\text{SA (unit/mg protein)} = \frac{\text{Protease activity}}{\text{Protein concentration}}$$

$$\text{PF} = \frac{SA_t}{SA_i}$$

where SA_t is the SA of the protease rich phase and SA_i is the initial SA ofthe crude extract or fraction before being partitioned.

The CPE/*t*-butanol ratio yielding the highest enzyme recovery and purity was chosen. The selected TPP fraction was used for ATPS.

3.4.4 Aqueous two-phase systems (ATPS)

ATPS was prepared in 10-mL centrifuge tubes by adding the different amounts of PEG and salts together with the selected TPP fraction according to the method of Klomklao *et al.* (2005).

3.4.4.1 Effect of salts on partitioning of protease in TPP fraction

To study the effect of salts on partitioning of the protease in TPP fraction, ATPS containing different salts, ammonium sulfate ($(NH_4)_2SO_4$), sodium citrate ($Na_3C_6H_5O_7$), magnesium sulfate ($MgSO_4$) and dipotassium hydrogen phosphate (K_2HPO_4), at different concentrations (15, 20 and 25%, w/w) with 20% PEG1000 were used. One mL of TPP fraction (1 mg protein/mL) was added into the system. Distilled water was used to adjust the system to obtain the final weight of 5 g. The mixtures were mixed continuously for 3 min using a vortex mixer (G-560E, Vortex-Genie 2, Scientific Industries, Inc., Bohemia, NY, USA). Phase separation was achieved by centrifugation for 5 min at 2000×*g*. Top phase was carefully separated using a pasteur pipette and the interface of each tube was discarded. Volumes of the separated phases, top and bottom phases, were measured. Aliquots from each phase were taken for protease assay and protein determination. Yield, SA and PF were then calculated.

Additionally partition coefficient (K_P) and volume ratio (V_R) were also calculated using the following equations:

$$K_P = \frac{C_T}{C_B}$$

where C_T and C_B are concentrations of protein in top and bottom phase, respectively.

$$V_R = \frac{V_T}{V_B}$$

where V_T and V_B are the volume of top and bottom phase, respectively.

Based on purity and yield, the appropriate salt in ATPS rendering the most effective partitioning was chosen for further study.

3.4.4.2 Effect of MW and concentration of PEG on partitioning of protease in TTP fraction

Effect of PEG1000, PEG2000 and PEG4000 at different concentrations (15, 20 and 25%, w/w) on partitioning of proteases in TPP fraction was studied in the presence of salt with the type and concentration showing the highest yield and purity. Partitioning was performed as described previously. ATPS rendering the most effective partitioning of protease was chosen for further study.

3.4.5 Back extraction (BE)

BE was used to partition the protease in PEG rich phase to aqueous salt rich phase following the method of Malpiedi *et al.* (2011) with a slight modification. Systems containing 25% PEG 4000 or 25% PEG 8000 in the presence of $MgSO_4$ at different final concentrations (5, 10, 15 and 20%, w/w) were used. One gram of the selected ATPS fraction was added into the prepared systems. Distilled water was used to adjust the system to obtain the final weight of 5 g. The mixtures were mixed continuously for 3 min using a vortex mixer. Phase separation was achieved by centrifugation for 5 min at 2000×*g*.The lower aqueous layer was collected and dialyzed against 50 volumes of distilled water overnight at 4°C with 4 changes of distilled water. After dialysis, the samples were determined for protease activity and total protein content. Yield, SA and PF were calculated. BE fraction with the highest purity and yield was used for hydrolysis study.

3.4.6 Enzyme assay

Protease activity was determined using BAPNA as a substrate according to the method of Khantaphant and Benjakul (2008). Sample (200 µL) was mixed with 200 µL of distilled water and 1000 µL of reaction buffer (50 mM Tris–HCl buffer, pH 8.0, containing 10 mM $CaCl_2$). The reaction was initiated by adding 200 µL of 2 mg/mL BAPNA to the reaction mixture. After incubation for 20 min at 60°C, 200 µL of 30% acetic acid (v/v) were added to terminate the reaction. Production of *p*-nitroaniline was measured by monitoring the absorbance of reaction mixture at 410 nm (A_{410}). A blank was conducted in the same manner except that

sample was added after addition of 30% acetic acid. One unit was defined as the amount causing an increase of 0.1 in A_{410} per min.

3.4.7 Characterization of recovered proteases

3.4.7.1 Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS–PAGE) andsubstrate-gel electrophoresis

SDS–PAGE of fractionated proteases was carried out according to the method of Laemmli (1970) using 12% separating gel and 4% stacking gel. CPE, TPP, ATPS and BE fractions were mixed with sample buffer (0.125 M Tris–HCl, pH 6.8, containing 4% SDS and 20% (v/v) glycerol) at a ratio of 2:1 (v/v). Proteins (4 µg) were loaded onto the gel. The electrophoresis was run at a constant current of 15 mA per gel by a Mini-Protein II Cell apparatus (Bio-Rad, Hercules, CA, USA). Proteins were stained with 0.05% Coomassie Blue G-250 in 15% methanol and 5% acetic acid dissolved in water and 0.076% formaldehyde. Destaining was performed using 40% methanol and 10% acetic acid.

Substrate-gel electrophoresis was performed after electrophoresis. The gels were immersed in 100 mL of 50 mM Tris–HCl buffer, pH 7.5, containing 2% casein (w/v) for 1 h at 0°C with a gentle agitation. Thereafter, the gels were transferred to 2% casein (w/v) in 50 mM Tris–HCl buffer. The mixture was incubated at pH 8 (60°C) for 15 min with continuous agitation. The gels were rinsed with distilled water and then stained with 0.05% Coomassie Blue R-250 in 15% methanol and 5% acetic acid. Thereafter, gels were destained in 30% methanol and 10% acetic acid. Proteolytic activity was indicated by the development of a clear zone on a blue background.

3.4.7.2 Hydrolysis of fish gelatin

Commercial fish gelatin powder was mixed with 50 mM Tris-HCl buffer (pH 8.0) to obtain the concentration of 2% (w/v). Gelatin solution was incubated at 60°C for 10 min. BE fraction with activity of 5 and 10 unit/g gelatin was added into gelatin solution to initiate hydrolytic reaction. At hydrolysis time designated (0, 5, 10, 15, 20, 30, 40, 60, 90 and 120 min), 1 mL of sample was taken and mixed with 1 mL of 1% SDS solution (90°C) before placing in a water bath

(model W350, Memmert, Schwabach, Germany) at 90°C for 15 min to inactivate protease.

The degree of hydrolysis (DH) of gelatin hydrolysate was analyzed according to the method of Benjakul and Morrissey (1997). The samples (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 water bath at 50°C for 30 min in dark. To terminate the reaction, 2.0 mL of 0.1 M sodium sulfite were added. The mixture was cooled for 15 min at room temperature. The absorbance was measured at 420 nm and α-amino acid content was calculated and expressed in terms of *L*-leucine. DH was calculated as follows:

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

where L_t is the amount of α-amino acid released at time t. L_0 is the amount of α-amino acid in original gelatin solution. L_{max} is the total α-amino acid in original gelatin solution obtained after acid hydrolysis with 6 N HCl at 100°C for 24 h.

3.4.8 Protein determination

Protein concentration was measured by the Bradford method using BSA as a protein standard (Bradford, 1976).

3.4.9 Statistical analysis

Experiments were run in triplicate using three different lots of samples. Data were subjected to analysis of variance (ANOVA). Comparison of means was carried out by Duncan's multiple range tests. For pair comparison, T-test was used (Steel and Torrie, 1980). Statistical analysis was performed using the Statistical Package for Social Science (SPSS 11.0 for windows, SPSS Inc., Chicago, IL, USA).

3.5 Results and discussion

3.5.1 Use of TPP for partitioning of proteases

TPP systems with various CPE to*t*-butanol ratios (1.0:0.5, 1.0:1.0, 1.0:1.5 and 1.0:2.0) in the presence of ammonium sulfate (30% saturation) were prepared (Table 11). The highest PF (2.6-fold) and yield (76.0%) were obtained in the

middle phase or interphase of TPP system with the CPE/*t*-butanol ratio of 1:1 (p<0.05). When CPE/*t*-butanol ratio of 1:0.5 was used the highest yield was found in the bottom phase (p<0.05). Nevertheless, very low yields were obtained in the bottom phase when the ratios above 1:0.5 were used. When considering PF, it was noted that the middle phase of CPE/*t*-butanol (1:1) system rendered the recovered proteases with the highest PF (p<0.01). PF was generally decreased as the CPE/*t*-butanol ratio increased. The results suggested that the volume of *t*-butanol and concentration ammonium sulfate in the TTP system played a key role in partitioning the target proteases. When crude polygalacturonase (pH 6.6) was saturated to 30% $(NH_4)_2SO_4$(w/v) in the presence of *t*-butanol at a ratio of 1:1 (v/v) at 25°C, 25.5% recovery with a PF of 6.7 was achieved. The extract to *t*-butanol ratio of 1:1 in the presence of $(NH_4)_2SO_4$ at 30% saturation was the optimal condition for partitioning of *α*-galactosidase from fermented media of *Aspergillus oryzae* (Dhananjay and Mulimani, 2009).

For TPP system, the middle phase or interphase showed the lower volumes than the bottom phase. As a result, the proteases were more likely concentrated in the interphase. In the present study, no proteases were detected in the top phase of proteases. The result suggested that proteases, which were polar in nature might not be able to migrate to *t*-butanol top phase. Additionally, proteases might be denatured in the solvent. Alcohol including *t*-butanol could bind water molecules, in which the water was removed from proteases. As a consequence, the conformational change could take place with the loss in activity. Therefore, CPE/*t*-butanol ratio of 1:1 was selected as the optimal ratio for partitioning of protease from CPE.

Table 11 TPP for portioning of proteases from the hepatopancreas of Pacific white shrimp

Phases	CPE/ *t*-butanol ratio (v/v)	Volumes (ml)	Protein (mg/ml)	Total protein (mg)	Activity (Unit/ml)	Total activity (Unit)	Specific activity (Unit/mg protein)	PF	%Yield
CPE		15.0±0.1[*a**]	7.03±0.12[a]	105.45±0.36[a]	6073.7±56.2[c]	91105.1±157.8[a]	864.0±55.5[f]	1.00±0.00[f]	100.00±0.00[a]
Middle	1:0.5	5.8±0.1[i]	1.22±0.15[f]	7.02±0.45[g]	1301.7±66.3[f]	7484.6±185.0[f]	1066.2±34.4[e]	1.23±0.02[e]	8.22±1.00[f]
Bottom	1:0.5	25.0±0.1[d]	1.63±0.18[e]	40.75±0.54[c]	3032.0±45.7[e]	75800.0±136.8[b]	1860.1±32.6[c]	2.15±0.01[c]	83.20±1.34[b]
Middle	1:1	7.2±0.1[h]	4.22±0.15[b]	30.38±0.45[d]	9612.7±185.3[a]	69211.2±555.7[d]	2278.2±44.2[a]	2.64±0.02[a]	75.97±1.45[d]
Bottom	1:1	24.0±0.0[e]	0.46±0.10	10.98±0.30[e]	27.9±10.2[g]	666.0±30.7[g]	60.7±18.6[g]	0.07±0.01[g]	0.73±0.03[g]
Middle	1:1.5	9.2±0.1[g]	3.40±0.02[c]	31.17±0.48[d]	6834.0±234.6[b]	62667.8±703.7[e]	2010.5±76.6[b]	2.33±0.03[b]	68.76±01.22[e]
Bottom	1:1.5	32.3±0.1[c]	0.25±0.06[g]	8.08±0.38[f]	15.8±3.5[g]	510.2±10.4[h]	63.1±16.2[g]	0.07±0.01[g]	0.56±0.01[h]
Middle	1:2	16.8±0.1[f]	2.86±0.11[d]	48.05±0.33[b]	4257.8±257.3[d]	71530.2±771.9c	1488.3±45.1[d]	1.72±0.01[d]	78.51±1.33[c]
Bottom	1:2	35.0±0.1[b]	0.19±0.07[g]	6.65±0.66[g]	7.9±1.3[h]	278.0±3.8[i]	41.8±13.0[g]	0.05±0.01[h]	0.31±0.01[i]

* Means ± SD (*n*=3). TPP was performed in the presence of $(NH_4)_2SO_4$ (30% saturation).

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

3.5.2 Use of ATPS for partitioning of proteases

Table 12 ATPS for partitioning of proteases from the hepatopancreas of Pacific white shrimp as affected by salts with different types and concentrations

Phase composition (%, W/W)	V_R	K_P	SA	PF†	Yield (%)
20% PEG 1000 + 15% $(NH_4)_2SO_4$	1.14±0.01^{*j**}	6.75±0.22^{j}	4445.3±29.0^{f}	1.45±0.01^{f} (5.15±0.03^{F})***	92.39±0.60^{j} (70.16±0.46^{J})
20% PEG 1000 + 20% $(NH_4)_2SO_4$	0.18±0.01^{a}	4.54±0.06^{e}	4954.1±36.1^{h}	1.62±0.01^{h} (5.73±0.04^{H})	97.82±0.71^{i} (74.16±0.54^{I})
20% PEG 1000 + 25% $(NH_4)_2SO_4$	0.62±0.00^{b}	3.72±0.24^{d}	4722.9±21.0^{g}	1.54±0.01^{g} (5.47±0.02^{G})	82.89±0.37^{g} (62.97±0.27^{G})
20% PEG 1000 + 15% $Na_3C_6H_5O_7$	0.93±0.01^{e}	5.17±0.9^{g}	3703.4±46.7^{d}	1.21±0.02^{d} (4.29±0.05^{D})	56.20±0.71^{d} (42.69±0.54^{D})
20% PEG 1000 + 20% $Na_3C_6H_5O_7$	1.04±0.01^{g}	5.67±0.20^{h}	4221.0±33.9^{e}	1.38±0.01^{e} (4.89±0.04^{E})	95.96±0.77^{k} (72.90±0.59^{K})
20% PEG 1000 + 25% $Na_3C_6H_5O_7$	0.72±0.00^{d}	4.77±0.05^{f}	4443.5±24.6^{f}	1.45±0.01^{f} (5.14±0.03^{F})	78.06±0.43^{e} (59.30±0.33^{E})
20% PEG 1000 + 15% $MgSO_4$	4.50±0.02^{l}	0.60±0.02^{a}	5036.0±50.5^{i}	1.65±0.02^{h} (5.83±0.06^{H})	80.10±0.80^{f} (60.85±0.61^{F})
20% PEG 1000 + 20% $MgSO_4$	1.41±0.02^{k}	1.53±0.07^{b}	5469.5±27.1^{j}	1.79±0.01^{i} (6.33±0.03^{I})	84.43±0.42^{h} (64.14±0.32^{H})
20% PEG 1000 + 25% $MgSO_4$	1.11±0.01^{i}	1.88±0.16^{c}	6300.1±13.7^{k}	2.06±0.00^{j} (7.29±0.02^{J})	86.25±0.19^{i} (65.52±0.14^{I})
20% PEG 1000 + 15% K_2HPO_4	1.10±0.00^{h}	7.76±0.10^{k}	1401.4±16.1^{b}	0.46±0.01^{b} (1.62±0.02^{B})	27.95±0.32^{c} (21.23±0.24^{C})
20% PEG 1000 + 15% K_2HPO_4	0.96±0.00^{f}	6.10±0.15^{i}	1290.6±23.7^{a}	0.42±0.01^{a} (1.49±0.03^{A})	24.25±0.45^{a} (18.42±0.34^{A})
20% PEG 1000 + 15% K_2HPO_4	0.70±0.01^{c}	3.97±0.49^{d}	1587.4±25.1^{c}	0.52±0.01^{c} (1.84±0.03^{C})	26.23±0.41^{b} (19.92±0.32^{B})

V_R: volume ratio (Upper/lower); K_P: partition coefficient of protein in the upper phase; SA: specific activity (unit/mg protein); PF: purification factor; Yield: activity recovery yield.

* Means ± SD (n=3). ** Different lowercase or uppercase superscripts in the same column indicate the significant differences (P<0.05). *** Values in the parenthesis were expressed, relative to those of CPE.

† Based on TTP fraction.

3.5.2.1 Effect of salts with different types and concentrations

Partitioning of protease from TTP fraction was carried out using several biphasic systems of 20% PEG1000 containing different salts including

$(NH_4)_2SO_4$, $Na_3C_6H_5O_7$, $MgSO_4$and K_2HPO_4at various concentrations (Table 12) After phase separation, two phases were obtained. Those included PEG-rich top phase and salt-rich lower phase. For all ATPS studied, the proteases were partitioned predominantly in the PEG-rich top phase, principally those with hydrophobic characteristics. In general, negatively charged proteins prefer the upper phase in PEG–salt systems, whilst positively charged proteins normally partition selectively to the bottom phase (Klomklao *et al.*, 2005). Reh *et al.* (2007) reported that the transfer of proteins, trypsin and *α*-chymotrypsin to top phase was associated with negative enthalpic and entropic changes. Hence, most hepatopancreas proteases partitioned in the top phase might be negatively charged.SA, PF and yield of protease obtained from PEG1000–salt systems depended on the types of salt used. The phase system containing $MgSO_4$ generally showed the superior partitioning efficiency to those containing other salts. A phase system containing 20% PEG1000 and 25% $MgSO_4$ gave the highest SA (6300 units/mg protein). Fraction obtained had PF of 2.1 and 86.2-fold, compared with TPP fraction and CPE, respectively. This result was is agreement with Klomklao *et al.* (2005) who found that ATPS comprising PEG1000 (15%, w/w) and $MgSO_4$ (20%, w/w) provided the best condition for the maximum partitioning of the protease from tuna spleen into the top phase and gave high SA and PF. It was noted that ATPS containing $(NH_4)_2SO_4$ showed the highest yield (82.89-92.39%). Nevertheless, PF was generally lower than that of system containing $MgSO_4$**.** Amongst all systems used, those with K_2HPO_4 rendered the lowest yield and PF.

Partition coefficient (*K*) is used to characterize the protein distribution in ATPS. Protein partitioning is reported as K_P (Klomklao *et al.*, 2005; Nalinanon *et al.*, 2009). ATPS with 15, 20 and 25% $MgSO_4$ exhibited the lower K_P (0.60, 1.53 and 1.88, respectively), indicating that the contaminant proteins, nucleic acid and other undesirable components more likely shifted to the lower phase. K_P obtained from the systems containing $(NH_4)_2SO_4$ or K_2HPO_4 were much higher than those found for the system with $MgSO_4$. High K_P was generally related with the lower PF. V_R were found to vary with type and concentration of salts used. V_R of 1.11 was obtained for ATPS having 20% PEG1000 and 25% $MgSO_4$. Thus, the extraction conditions employed determined the partitioning efficacy of the desired protease to the top phase and

partition the contaminating enzymes and proteins to the opposite phase. Due to the high yield and SA, ATPS with 25% $MgSO_4$ was selected for further study**.**

3.5.2.2 Effect of PEG with different MW and concentrations

Partitioning of protease from TTP fraction using ATPS containing PEG with varying MW and concentrations in the presence of 25% $MgSO_4$ is shown in Table 13. In general, PEG with higher MW tended to yield the proteases with lower PF and yield. When the same PEG was used, PF and yield varied, depending upon level of PEG. ATPS of 15% PEG1000–25% $MgSO_4$ gave the highest PF (2.4-fold) with 86.2% yield for partitioning of protease from TTP fraction (p<0.05). When comparing with CPE, PF of 8.59 was obtained. Partitioning of protease in PEG–$MgSO_4$ system was strongly dependent on the MW of the PEG (Klomklao *et al.*, 2005). Reh *et al.* (2007) reported that most proteases were partitioned to the top phase when systems contained low MW PEG. The influence of the MW of PEG on protein partitioning can be explained on the basis of Flory Huggins theory for polymers. For ATPS formed by PEG of low MW (600–3350 kDa), the proteins transfer to the top phase is enthalpically driven, mainly due to a strong interaction between PEG and the protein (Tubío *et al.*, 2007). ATPS comprising PEG 600 (20.62%, w/w) and sodium tartrate (12.85 %, w/w) provided the better condition for the maximum partitioning of trypsinogen (TRPz) and α-chymotrypsinogen (ChTRPz) from bovine pancreas into the top phase when compared with PEG 2000, 4000 and 6000 (Malpiedi *et al.*, 2011). With ATPS, other pancreatic homogenate components – cell debris, nucleic acids and lipids were either partitioned into the bottom phase or precipitated at the interphase (Malpiedi *et al.*, 2011). Therefore, PEG1000 was a suitable polymer for partitioning of protease in TTP fraction as indicated by the higher PF than PEG with higher MW.

The K_P obtained from most systems was higher than 1, indicating that most of the protein components from TTP fraction preferably partitioned to the top phase of ATPS. The V_R of the system ranged from 0.75 to 1.29. Most of the systems yielded V_R higher than 1.0, indicating that the volume fraction was distributed in the top phase rather than in the bottom phase.

Table 13 ATPS for partitioning of proteases from the hepatopancreas of Pacific white shrimp as affected by PEG with different molecular weight and concentrations

Phase composition (%, W/W)	V_R	K_P	SA	PF†	Yield (%)
15% PEG 1000 + 25% $MgSO_4$	0.82±0.01^{*c**}	5.37±0.28^{g}	7423.0±69.3^{i}	2.43±0.02^{h} (8.59±0.08^{H})***	86.23±0.80^{h} (65.51±0.61^{H})
20% PEG 1000 + 25% $MgSO_4$	1.13±0.01^{f}	1.11±0.23^{h}	7153.6±78.4^{h}	2.34±0.03^{g} (8.28±0.09^{G})	82.99±0.91^{g} (63.04±0.69^{G})
25% PEG 1000 + 25% $MgSO_4$	1.29±0.02^{h}	0.95±0.09^{a}	5280.4±59.6^{d}	1.73±0.02^{d} (6.11±0.07^{D})	39.72±0.45^{c} (30.17±0.34^{C})
15% PEG 2000 + 25% $MgSO_4$	0.80±0.01^{b}	1.30±0.01^{d}	5713.3±43.5^{f}	1.87±0.01^{e} (6.61±0.05^{E})	56.86±0.43^{e} (43.19±0.33^{E})
20% PEG 2000 + 25% $MgSO_4$	1.11±0.01^{e}	1.86±0.08^{f}	5686.8±66.3^{e}	1.86±0.02^{e} (6.65±0.08^{E})	66.65±0.78^{f} (50.63±0.59^{H})
25% PEG 2000 + 25% $MgSO_4$	1.24±0.02^{g}	1.14±0.28^{b}	2734.8±63.2^{c}	0.89±0.02^{c} (3.17±0.07^{C})	28.94±0.67^{b} (21.99±0.51^{B})
15% PEG 4000 + 25% $MgSO_4$	0.75±0.01^{a}	0.96±0.12^{a}	6703.1±37.2^{g}	2.19±0.01^{f} (7.76±0.04^{F})	50.76±0.28^{d} (38.56±0.21^{D})
20% PEG 4000 + 25% $MgSO_4$	1.02±0.01^{d}	1.27±0.05^{c}	1973±44.3^{a}	0.65±0.01^{a} (2.28±0.05^{A})	1.69±0.38^{a} (12.89±0.29^{A})
25% PEG 4000 + 20% $MgSO_4$	1.27±0.01^{h}	1.63±0.18^{e}	2651.1±39.6^{b}	0.87±0.00^{b} (3.07±0.05^{B})	29.07±0.43^{b} (22.09±0.33^{H})

V_R: volume ratio (Upper/lower); K_P: partition coefficient of protein in the upper phase; SA: specific activity (unit/mg protein); PF: purification factor; Yield: activity recovery yield.

* Means ± SD (n=3). ** Different lowercase or uppercase superscripts in the same column indicate the significant differences (P<0.05). *** Values in the parenthesis were expressed, relative to those of CPE.

† Based on TTP fraction.

3.5.3 Use of BE for partitioning of proteases

After ATPS was implemented, various methods, such as ultra-filtration and gel chromatography, were used to remove the PEG from the top phase (Loc and Mien, 2010). In the present study, ATPS containing 25% PEG4000 and PEG8000 in the presence of $MgSO_4$ at different concentrations were used for back extraction as shown in Table 14. BE using ATPS containing 25% PEG8000 and10% $MgSO_4$ gave the highest PF (6.4-fold) with 70.4% yield (p<0.05). PF of 9.94 was achieved,

compared with CPE. Yield of 46.16% was found, in comparison with total activity of CPE. In the presence of 5 or 10% $MgSO_4$, PEG4000 showed the lower PF and yield than PEG8000. However, with higher level of $MgSO_4$ (20%), lower yield and PF were found when PEG8000 was used. Therefore, PEG8000 was a suitable polymer for partitioning of protease to bottom salt phase as indicated by the higher SA and PF than PEG4000 (p<0.05).

Table 14 BE for partitioning of protease from the hepatopancreas of Pacific white shrimp as affected by PEG with different molecular weight and $MgSO_4$ at various concentrations

Phase composition (%, W/W)	Total protein (mg)	Total activity (units)	Specific activity (units/mg)	PF†	Yield (%)
25% PEG4000 + 5% $MgSO_4$	0.19±0.02^{*c**}	928.38±0.23^{b}	4840.8±55.3^{a}	3.61±0.03^{a} (5.60±0.14)^{A***}	30.34±0.80^{b} (19.89±0.59)^{B}
25% PEG4000 + 10%$MgSO_4$	0.23±0.03^{c}	1788.82±0.12^{g}	7729.8±79.0^{e}	5.77±0.05^{f} (8.95±0.21)^{F}	58.47±0.67^{f} (38.32±0.34)^{F}
25% PEG4000 + 15%$MgSO_4$	0.14±0.02^{a}	934.76±0.25^{c}	6692.1±68.1^{d}	4.99±0.04^{e} (7.75±0.21)^{E}	30.55±0.78^{b} (20.02±0.56)^{B}
25% PEG4000 + 20%$MgSO_4$	0.21±0.03^{c}	1396.36±0.26^{f}	6603.4±78.2^{d}	4.93±0.05^{e} (7.64±0.24)^{E}	45.64±0.95^{e} (29.91±0.76)^{E}
25% PEG8000 + 5%$MgSO_4$	0.20±0.03^{c}	1221.33±0.15^{d}	6145.54±89.3^{c}	4.58±0.06^{d} (7.11±0.30)^{D}	39.92±0.66^{c} (26.16±0.34)^{C}
25% PEG8000 + 10%$MgSO_4$	0.25±0.04^{c}	2154.96±0.20^{h}	8586.2±49.7^{f}	6.40±0.05^{g} (9.94 ±0.25)^{G}	70.43±0.47^{g} (46.16±0.25)^{G}
25% PEG8000 + 15%$MgSO_4$	0.10±0.05^{a}	567.30±0.25^{a}	5848.5±99.4^{b}	4.36±0.03^{b} (6.77±0.13)^{B}	18.54±0.75^{a} (12.15±0.55)^{A}
25% PEG8000 + 20%$MgSO_4$	0.21±0.04^{c}	1285.12±0.24^{e}	6249.4±77.5^{c}	4.66±0.02^{c} (7.23±0.12)^{C}	42.00±0.87^{d} (27.53±0.65)^{D}

* Means ± SD (n=3).

** Different lowercase or uppercase superscripts in the same column indicate the significant differences (p<0.05).

*** Values in the parenthesis were expressed, relative to those of CPE.

† Based on ATPS fraction.

PEG of the highest MW (PEG 8000) excludes the protein from the top phase, driven by an entropically unfavorable term (Tubío *et al.*, 2007). For systems

containing PEG of low MW, the free energy changes adopt negative values (enthalpic changes overcome entropic changes), whilst the opposite behavior ($\triangle$G transfer > 0) occurs for ATPS with PEG of higher MW (Tubío *et al.*, 2007). Malpiedi *et al.* (2011) reported that BE of pancreatic TRPz and ChTRPz from bovine pancreasin the PEG-rich top phase was achieved using high MW PEG (PEG 6000; 3.70%, w/w). BE induced both zymogens to transfer to the salt enriched phase and induced the formation of a new two phase system, in which BE of both zymogen took place (Baskir *et al.*, 1989). In the present study, ATPS containing 25% PEG8000 and 10% $MgSO_4$ was shown to be the effective BE to transfer protease to the salt phase.

3.5.4 Protein pattern and activity staining of protease from hepatopancreas partitioned with combined partitioning system

Protein patterns of CPE and TTP, ATPS, BE fractions are shown in Figure 5A. CPE contained a variety of proteins with different MW. CPE contained proteins with MW of 36 and 26 kDa as the major proteins. Proteins with MW of 119, 98, 84, 69, 54, 50, 23 and 22 kDa were also found in CPE. However, a large number of proteins were removed after partitioning with ATPS and BE, particularly proteins with higher or lower MW. It was noted that proteins with MW of 119 and 84 kDa were removed after being partitioned with TTP. Also protein with MW of 23 kDa was eliminated after ATPS and BE were used. As a result, a higher purity of proteases was obtained. When the proteins or enzymes differ significantly in their structural properties from others, the partitioning can be carried out successfully (Klomklao *et al.*, 2005).

The proteases in CPE and TTP ATPS and BE fractions were determined by SDS-substrate polyacrylamide gels (Figure 5 (B)). The apparent MWs of the major activity bands were estimated to be 36 and 26 kDa for all fractions. Slightly greater band intensity in ATPS and BE fraction was observed, suggesting the higher specific activity of protease loaded into the gel. Proteases in hepatopancreas were more likely trypsin or trypsin-like enzyme, which had MW in the range of trypsin previously reported. Trypsins purified from black tiger prawn had MW of 27–29 kDa (Lu *et al.*, 1990). Trypsin A, B, C and D from the hepatopancreas of crawfish had MW of 35.0, 41.2, 37.9 and 39.5 kDa (Jeong *et al.*, 2000) and trypsin from

hepatopancreas of shrimp *Penausindicus* had MW of 36 kDa (Honjo *et al.*, 1990). Trypsin from Antarctic krill processing had MW between 32 and 33 kDa. The optimal pH and temperature of trypsin activity were pH 8 and 60°C, respectively (Bustos *et al.*, 1999). Trypsin from the hepatopancreas of *Sepia officinalis* exhibited the maximal activity at 60°C, and pH 8 using BAPNA as a substrate (Balti *et al.*, 2009). Additionally, trypsin from digestive gland of the shrimp (*Penaeus japonicas*) had the MW of 25 kDa with the optimal pH and temperature of 8 to 8.3 and 60°C, respectively (Galgani *et al.*, 1985).

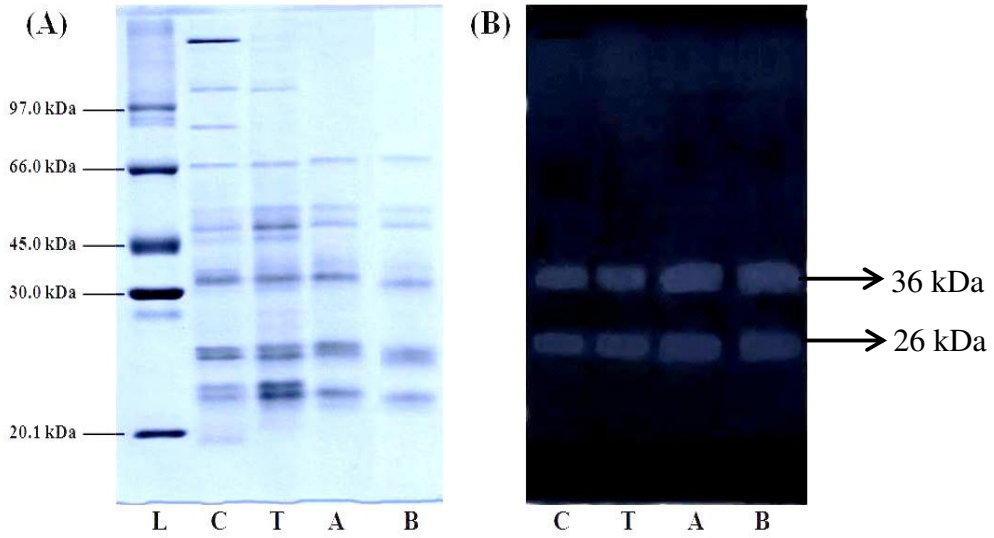

Figure 5 SDS-PAGE (A) and activity staining (B) of CPE, TPP, ATPS and BE fractions from hepatopancreas of Pacific white shrimp. L: low-molecular-weight standard; C: crude protease extract; T: TPP fraction (The middle phase of TPP containing CPE: *t*-butanol (1: 0.5) and 30% $(NH_4)_2SO_4$); A: ATPS fraction containing15% PEG1000–25% $MgSO_4$; B: BE containing 25% PEG8000–10% $MgSO_4$.

3.5.5 Hydrolysis of fish gelatin using partionated proteases

When fish gelatin was hydrolyzed using BE fraction (5 and 10 unit BAPNA), a rapid hydrolysis was found within the first 30-40 min, followed by a slower hydrolysis rate up to 120 min (Figure 6). The rapid hydrolysis in the initial phase indicated that a large number of peptide bonds were hydrolyzed (Shahidi *et al.*, 1995). Thereafter, the hydrolysis rate was decreased, mainly due to a decrease in available hydrolysis sites, enzyme autodigestion and/or product inhibition (Kristinsson and Rasco, 2000). At the same hydrolysis time, higher DH was observed with higher activity level of BE fraction used. Gelatin hydrolysate with bioactive activities has been produced with the aid of commercial protease, particularly from microorganism such as Alcalase and Flavouryme (Phanturat *et al.*, 2010). Thus, proteases recovered from hepatopancreas using the combined partitioning systems could be an alternative potential aid for production of gelatin hydrolysate, in which the cost of enzyme could be reduced.

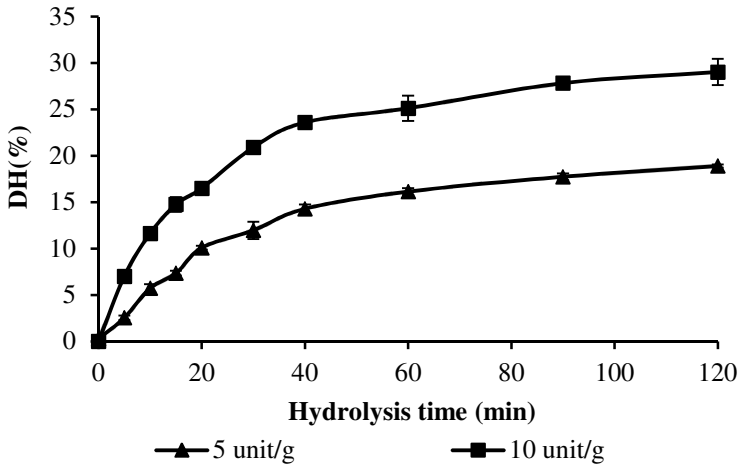

Figure 6 Changes in degree of hydrolysis (DH) of fish gelatin during hydrolysis with BE fraction of hepatopancreas from Pacific white shrimp. The hydrolytic reaction was performed at pH 8.0, 60^{o}C. Bars represent the SD (n=3).

3.6 Conclusion

The combined partitioning systems including TPP, ATPS and BE effectively recovered the proteases from hepatopancrease of Pacific white shrimp. TPP with CPE/*t*-butanol ratio of 1:1 was firstly implemented. ATPS containing 15% PEG1000–25% $MgSO_4$ was further used and BE including 25% PEG8000–10% $MgSO_4$ was applied as the last step. The highest PF (9.94-fold) with 46.16% yield were obtained. Protease with MW of 36 and 26 kDa were dominant and were able to hydrolyse fish gelatin in dose-dependent manner.

3.7 References

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CHAPTER 4

Compositions and yield of lipids extracted from hepatopancreas of Pacific white shrimp (*Litopenaeus vannamei*) as affected by prior autolysis

4.1 Abstract

Compositions and yield of lipids extracted from hepatopancreas of Pacific white shrimp (*Litopenaeus vannamei*) subjected to autolysis at 60°C for different times (0, 30, 60, 90 120 and 150 min) were investigated. Extraction yield increased from 7.4% to 8.8% as autolysis time increased from 0 to 150 min. Coincidental increase in total carotenoid content was obtained with increasing autolysis time ($p<0.05$). The increases in thiobarbituric acid-reactive substances (TBARS) and *p*-anisidine value (AV) of lipids were noticeable when autolysis time increased ($p<0.05$). However, no changes in free fatty acid (FFA) content were observed within the first 60 min of autolysis ($p>0.05$), but subsequently increased up to 150 min. ($p<0.05$). No differences in fatty acid profiles of lipids extracted from hepatopancreas without and with 60 min prior autolysis were observed. Lipids extracted contained docosahexaenoic acid (DHA; C22:6 (n-3)) as the most abundant fatty acid, followed by eicosapentaenoic acid (EPA; C20:5 (n-3)). Therefore, prior autolysis at 60°C for 60 min increased the extraction yield without negative effect on lipid quality.

4.2 Introduction

Thailand has been the world's leading exporter of cultivated shrimp since the mid-90s, (Lebel *et al.*, 2010). White shrimp and white shrimp products are an economically important crustacean of Thailand. By the year of 2010, frozen white shrimp and white shrimp products were manufactured and exported, mostly to the USA and Japan, with a total amount of 407978 metric tons (Lebel *et al.*, 2010). During shrimp processing, a large amount of by-products including cephalothorax, shell, etc. are generated. Shrimp cephalothorax has been used as raw material for shrimp hydrolysate, shrimp flavorant, carotenoid and chitin/chitosan (Armenta-López

et al., 2002; Chakrabarti, 2002; De Holanda and Netto, 2006; Flores *et al.*, 2007). Lipids of shrimp head and shell from Indian white shrimp (*Penaeus indicus*) were extracted with a yield of 9.8% (dry weight basis) (Ravichandran *et al.*, 2009). Krill oil was also extracted from krill, a shrimp-like crustacean (Sampalis *et al.*, 2003). Similar to fish oil, krill oil is rich in long chain omega-3 polyunsaturated fatty acids, mainly docosahexaenoic acid (DHA) and eicosapentaenoic acid (EPA), which have been associated with a wide-range of health benefits to humans (Duan *et al.*, 2010; Matsumoto *et al.*, 2009). Furthermore, krill oil contains vitamin E, vitamin A, vitamin D and canthaxanthin. The antioxidant potency of krill oil was found to be 48-fold more potent than fish oil (Sampalis *et al.*, 2003). For shrimp shell, it has been intensively used for chitin and chitosan production (Percot *et al.*, 2003). Furthermore chitin/chitosan with bioactivity has been prepared (Dutta *et al.*, 2004). Carotenoprotein was also recovered from black tiger shrimp cephalothorax (Sowmya and Sachindra, 2012).

Among all shrimp products, there is currently an increasing demand for whole shrimp without hepatopancreas. Hepatopancreas is removed and recovered by a vacuum sucking machine. Hepatopancreas can be the major source of lipids, carotenoid, etc. (Clarke, 1979; Pacheco *et al.*, 2009). Additionally, shrimp hepatopancreas has been known to be an excellent source of proteases, especially trypsin (Oh *et al.*, 2000; Sriket *et al.*, 2012). Since hepatopancreas contains high amount of proteases, lipoproteins and carotenoprotein, autolysis mediated by endogenous proteases might increase the extraction yield of lipids as well as carotenoid. However, little information about lipids and carotenoids from Pacific white shrimp hepatopancreas has been reported. Therefore, the aim of the present study was to investigate the impact of autolysis on extraction efficiency and properties of lipids from the hepatopancreas of white shrimp, a by-product from a shrimp processing plant.

4.3 Objective

To investigate autolysis profile and the impact of autolysis on lipid separation from hepatopancreas of Pacific white shrimp.

4.4 Materials and methods

4.4.1 Chemicals

Palmitic acid, cupric acetate, *p*-anisidine, ammonium thiocyanate, cupric acetate and pyridine were purchased from Sigma (St. Louis. MO, USA). Trichloroacetic acid, anhydrous sodium sulfate, isooctane and ferrous chloride were obtained from Merck (Darmstadt, Germany). 2-Thiobarbituric acid and 1,1,3,3-tetramethoxypropane were procured from Fluka (Buchs, Switzerland). Methanol, ethanol, chloroform, petroleum ether, hydrochloric acid, sulfuric acid and ammonium thiocyanate were purchased from Lab-Scan (Bangkok, Thailand). Astaxanthin was obtained from Dr. Ehrenstorfer GmbH (Augsburg, Germany).

4.4.2 Collection and preparation of hepatopancreas from Pacific white shrimp

Hepatopancreas of Pacific white shrimp (*Litopenaeus vannamei*) was obtained from the Sea Wealth Frozen Food Co., Ltd. in Songkhla. Pooled hepatopancreas was placed in a polyethylene bag and transported on ice to the Department of Food Technology, Prince of Songkla University, Hat Yai, Songkhla within approximately 2 h. The samples were stored at -18°C until use, but the storage time was not longer than 1 month. Hepatopancreas was analyzed for moisture, ash, fat and protein contents according to the method of AOAC (2000). The values were expressed as % (wet weight basis). Prior to analyses or extraction, hepatopancreas was ground using a blender (Phillips, Guangzhou, China) for 30 s.

4.4.3 Autolysis study of hepatopancreas

Ground hepatopancreas (45 g) was placed in a 500 mL-beaker and incubated at different temperatures (30, 40, 50, 55, 60, 65, 70 and 80°C) for 30 min. After the designated incubation time, autolysis was terminated by addition of 5 mL of 50% trichloroacetic acid (TCA). The mixtures were homogenized at a speed of 11,000 rpm for 2 min using an IKA Labortechnik homogenizer (Selangor, Malaysia). Thereafter, the homogenate was centrifuged at 3000×g for 15 min using a refrigerated

centrifuge (Beckman Coulter, Avanti J-E Centrifuge, Fullerton, CA, USA). TCA-soluble peptides in the supernatant were determined using the Lowry's method (Lowry *et al.*, 1951).

4.4.4 Effect of autolysis time on extraction and properties of lipids

Hepatopancreas was incubated at the optimal temperature for different times (0, 30, 60, 90, 120 and 150 min). The samples were cooled suddenly in iced water, followed by lipid extraction. Lipid was extracted by the Bligh and Dyer method (Bligh & Dyer, 1959). Sample (25 g) was homogenised with 200 ml of a chloroform:methanol:distilled water mixture (50:100:50, v/v/v) at a speed of 9500 rpm for 2 min at 4 C. The homogenate was added with 50 ml of chloroform and homogenised at 9500 rpm for 1 min. Thereafter, 25 ml of distilled water were added and homogenized at the same speed for 30 s. The homogenate was centrifuged at 3000×g at 4 C for 15 min and transferred into a separating flask. The chloroform phase was drained off into the 125 ml Erlenmeyer flask containing 2–5 g of anhydrous sodium sulphate, shaken very well, and decanted into a round-bottom flask through a Whatman No.4 filter paper (Whatman International Ltd., Maidstone, England). The solvent was evaporated at 25 C using an EYELA rotary evaporator N-1000 (Tokyo Rikakikai, Co. Ltd, Tokyo, Japan) and the residual solvent was removed by nitrogen flushing.

The yield was calculated and expressed as the percentage of lipids extracted. Lipid samples were collected in a vial, flushed with nitrogen gas, sealed tightly and kept at 40 C until analysis.

4.4.5 Analyses

4.4.5.1 Measurement of total carotenoid content

Total carotenoid content in the lipid samples was determined according to the method of Saito and Regier (1971) with a slight modification. Lipid (30 mg) was mixed with 10 mL of petroleum ether and the mixture was allowed to stand for 30 min. The absorbance of the extract, appropriately diluted, was measured at 468 nm using a UV-1601 spectrophotometer (Shimadzu, Kyoto, Japan). The concentration (C)

of carotenoid in the sample was calculated using the equation given by Saito and Regier (1971) with a slight modification as follows:

$$C\ (\mu g/g\ lipid) = \frac{A_{468} \times \text{vol of extract} \times \text{dilution factor}}{0.2 \times \text{weight of sample used in gram}}$$

where 0.2 is the A468 of 1 µg /mL standard asthaxanthin

4.4.5.2 Measurement of peroxide value (PV)

Peroxide value was determined using the ferric thiocyanate method (Chaijan *et al.*, 2006). To 50 µL of lipid sample (10-fold dilution), 2.35 mL of 75% ethanol (v/v), 50 µL of 30% ammonium thiocyanate (w/v) and 50 µl of 20 mM ferrous chloride solution in 3.5% HCl (w/v) were added and mixed thoroughly. After 3 min, the absorbance of the colored solution was read at 500 nm using a spectrophotometer. Blank was prepared in the same manner, except the distilled water was used instead of ferrous chloride. PV was expressed as A_{500} after blank subtraction.

4.4.5.3 Measurement of thiobarbituric acid reactive substances (TBARS)

Thiobarbituric acid-reactive substances (TBARS) were determined as described by Buege and Aust (1978). Lipid sample (0.5 g) 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 boiling water (95–100°C) for 10 min to develop a pink color, cooled with running tap water and centrifuged at 3600 ×g at 25°C for 20 min. 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 was calculated and expressed as mg malonaldehyde/kg sample.

4.4.5.4 Measurement of *p*-anisidine value (AnV)

AnV of sample was analysed according to the method of AOCS (AOCS, 1990).

4.4.5.5 Measurement of free fatty acid

Free fatty acid (FFA) content, used as an index of hydrolysis, was determined according to the method of Lowry and Tinsley (1976). Lipid sample (0.1 g) was added with 5 mL of isooctane and swirled vigorously to dissolve the sample. The mixture was then added with 1 mL of 5% (w/v) cupric acetate-pyridine reagent, prepared by dissolving 5 g of the reagent grade cupric acetate in 100 mL of water, filtering and adjusting the pH to 6.0-6.2 using pyridine. The mixture was shaken vigorously for 90 sec using a Vortex-Genie2 mixer (Bohemia, NY, USA) and allowed to stand for 20 sec. The upper layer was subjected to absorbance measurement at 715 nm. A standard curve was prepared using palmitic acid in isooctane at concentrations ranging from 0 to 50 μmol/5 mL. FFA content was expressed as g FFA/100 g lipid.

4.4.5.6 Determination of Lipid classes

Lipid compositions were determined using a thin layer chromatography/ flame ionisation detection analyser (IATROSCAN® TLC/ FID Analyzer, IATRON Laboratories, Inc., Tokyo, Japan). The chromarods S-III (silica gel powder-coated Chromarod S-III, Iatron Laboratories Inc., Tokyo, Japan) were cleaned by soaking in 50% nitric acid solution in water and washed with tap water, distilled water and acetone. They were dried and scanned twice before use in order to remove possible contaminants from the rods. The lipid samples, dissolved in chloroform, were spotted on the rod. Lipid classes were separated using a double development procedure with the following solvent systems: n-hexane: diethyl ether: formic acid (50:20:0.3, v/v/v) for approximately 15 min followed by hexane: benzene (1:1, v/v) for approximately 30 min. The rods were then dried in an oven (105°C) for 10 min before being analyzed with the FID detector. The analyses were carried out under the following conditions: flow rate of hydrogen, 150 mL/min; flow rate of air,

700 mL/min; scan speed, 30 s/scan. Peak area was quantitated and expressed as % of total lipid.

4.4.5.7 Fatty acid profile

Fatty acid profile was determined as fatty acid methyl esters (FAMEs), which were prepared according to the method of AOAC (2000). FAMEs were injected to the gas chromatography (Shimadzu, Kyoto, Japan) equipped with the flame ionization detector (FID) at a split ratio of 1:20. A fused silica capillary column (30 m × 0.25 mm), coated with bonded polyglycol liquid phase, was used. The analytical conditions were: injection port temperature of 250 ºC and detector temperature of 270 ºC. The oven was programmed from 170 to 225 ºC at a rate of 1 ºC/min (no initial or final hold). Retention times of FAME standards were used to identify chromatographic peaks of the samples. Fatty acid content was calculated, based on the peak area ratio and expressed as g fatty acid/100 g lipid.

4.4.6 Statistical analysis

Experiments were run in triplicate using three different lots of samples. Data were subjected to analysis of variance (ANOVA). Comparison of means was carried out by Duncan's multiple range test. For pair comparison, T-test was used (Steel and Torrie, 1980). Statistical analysis was performed using the Statistical Package for Social Science (SPSS 11.0 for Windows, SPSS Inc., Chicago, IL, USA)

4.5 Results and discussion

4.5.1 Proximate composition

Hepatopancreas of Pacific white shrimp had high moisture content (71.9 ± 0.5%). Protein and lipid contents were 13.4 ± 0.3% and 10.7 ± 0.2%, respectively. Low ash content (2.1 ± 0.1%) was found in hepatopancreas. Based on a dry weight basis, hepatopancreas contained 47.7 ± 0.3% protein and 36.9 ± 0.2% lipid. The result indicated that hepatopancreas could serve as a good source of both protein and lipid. Ravichandran *et al.* (2009) reported that lipid content of shrimp head and shell from Indian white Shrimp (*P. indicus*) was 9.8% (% dry weight).

4.5.2 Effect of temperatures on autolysis of Pacific white shrimp hepatopancreas

Autolysis of Pacific white shrimp hepatopancreas at different temperatures (30–80°C) for 30 min is shown in Figure 7. Autolytic activity was highest at 60°C as indicated by the highest TCA-soluble peptide content (p<0.05). However, a decrease in autolytic activity was found when the temperature was above 60°C. At high temperature, unfolding or structural changes of proteases might occur, leading to a loss in activity. Sriket *et al.* (2011) reported that the highest proteolytic activity of crude protease extract from the hepatopancreas of freshwater prawn (*Macrobrachium rosenbergii*) was found at pH 7 and 60°C when casein was used as a substrate. Major proteases isolated from hepatopancreas of Northern shrimps (*Pandalus borealis*) were classified as serine proteases (Aoki *et al.*, 2003). Extract of *Penaeus vannamei* hepatopancreas consisted of trypsin and chymotrypsin (Ezquerra *et al.*, 1997). Trypsins from the pyloric ceca of jacopever (*Sebastes schlegelii*) had the optimum temperature of 60°C (Klomklao *et al.*, 2007). Thus, the maximized autolysis of hepatopancreas from Pacific white shrimp could be achieved at 60°C. With pronounced hydrolysis, lipoproteins could be hydrolyzed, thereby releasing more free lipids, which could be readily extracted.

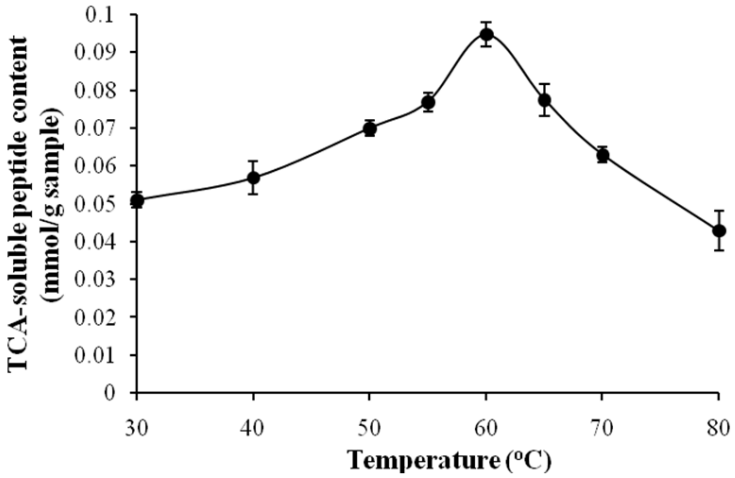

Figure 7 TCA-soluble peptide content oil in hepatopancreas of Pacific white shrimp incubated at different temperatures. Bars indicate standard deviations (n=3).

4.5.3 Effect of prior autolysis on lipid yield

As the autolysis time increased, yield of lipids from hepatopancreas of Pacific white shrimp increased (p<0.05) (Figure 8A). Yield of extracted lipids increased from 7.4% to 8.8%, when autolysis time increased from 0 to 150 min. When autolysis proceeded, proteins associated with lipids, e.g. lipoproteins, were cleaved to a higher extent. As a result, lipids were liberated from proteins. Therefore, the extraction efficiency became higher when autolysis mediated by endogenous proteases was performed. As a consequence, the cost of exogenous proteases could be reduced. In general, exogenous proteases have been used to hydrolyze tissue proteins, resulting in the release of oil from the protein matrix of marine byproducts. Lipids extracted from salmon (*Salmo salar*) heads by commercial protease had a yield of 19.6%, while the yield of lipids obtained from cooking method was lower (14.5%) (Gbogouri *et al.*, 2006). Linder *et al.* (2005) reported that oil (17.4%) obtained from salmon head after 2 h of hydrolysis with Alcalase 2.4 l was close to that obtained by the chemical extraction method (20%) (Linder *et al.*, 2005). Liaset *et al.* (2002) recovered 77% of total lipids from the salmon frames by enzymatic hydrolysis with Protamex®.

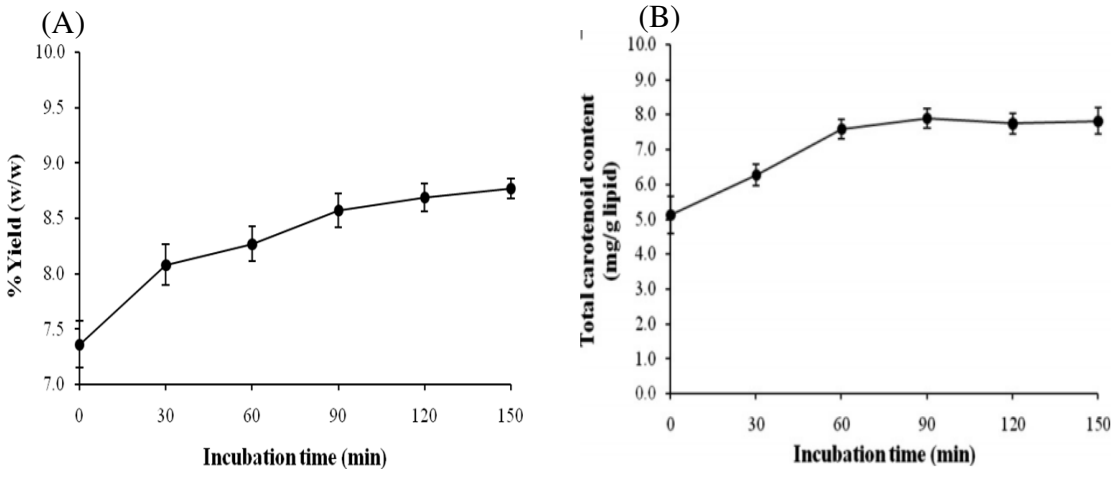

Figure 8 Yield (A) and carotenoid content (B) of lipid from hepatopancreas of Pacific white shrimp incubated at 60°C for different times. Bars indicate standard deviations (n=3).

4.5.4 Effect of prior autolysis on carotenoid content of lipids

Carotenoid contents of lipid from hepatopancreas of Pacific white shrimp as a function of autolysis time at 60°C are shown in Figure 8B. Carotenoid content of lipids from hepatopancreas increased as the autolysis time increased up to 60 min (p<0.05). No changes in carotenoid content were found during 60 and 150 min of autolysis (p>0.05). Total carotenoid content of 7.58 ± 0.29 mg/g lipid was obtained when lipids were extracted from hepatopancreas with prior autolysis for 60 min. Generally the complexes of carotenoids and proteins (carotenoproteins and carotenolipoproteins) were prevalent (Simpson and Haard, 1985). As a result, lipids containing carotenoid showed the red color. The red color of cooked crustaceans is produced by the release of the individual astaxanthin prosthetic groups from the carotenoproteins when denatured by heat (Lorenz and Cysewski, 2000).The carotenoid content in the wastes from Indian shrimps was found to vary from 35 to 153 µg/g depending on the species and the major pigments were astaxanthin and its esters (Sachindra *et al.*, 2006). The recovery of carotenoproteins from black tiger shrimp shells was maximized by the hydrolysis of shrimp shells using trypsin from bluefish (1.2 trypsin units/g shrimp shells) for 1 h at 25°C (Klomklao *et al.*, 2009). Carotenoprotein recovered contained 19.76% lipid and 87.91 mg total astaxanthin/g sample (Klomklao *et al.*, 2009).

4.5.5 Effect of prior autolysis on hydrolysis and oxidation of lipids

4.5.5.1 Peroxide value (PV)

Changes in PV of lipids from hepatopancreas of Pacific white shrimp autolyzed at 60°C for different times are depicted in Figure 9A. PV slightly increased within the first 30 min. of autolysis (p<0.05). After 60 min of autolysis, an increase in PV was obtainedup to 90 min (p<0.05). Thereafter, no difference in PV of lipids, was obtained from hepatopancreas autolyzed for 90–150 min (p>0.05). The result suggested that lipid oxidation took place in lipids from hepatopancreas during autolysis at 60°C for an extended time. At high temperature (60°C) used for autolysis, fatty acids likely underwent more oxidation, especially as the autolysis time

increased. The formation of autoxidation products during the induction period is slow at low temperature (Choe and Min, 2006). The concentration of the hydroperoxides generally increases until the advanced stages of oxidation. Formation of hydroperoxide is influenced by heat and some other factors such as light, oxygen, metal and fatty acid composition of oil (Shahidi and Spurvey, 1996). In the present study, it was noted that some hydroperoxide generated might undergo decomposition at autolysis temperature (60°C). The hydroperoxide decomposition rate of crude herring oil stored at 50°C in the dark was higher than the formation rate of hydroperoxide. The reverse phenomena were observed in the same crude herring oil at 0 or 20°C in the dark (Aidos *et al.*, 2002).

4.5.5.2 TBARS

Changes in TBARS of lipid from hepatopancreas of Pacific white shrimp subjected to autolysis at 60°C for different times are shown in Fig. 9B. TBARS value of lipid from hepatopancreas increased within the first 60 min ($p<0.05$). Thereafter, the higher increase was found during 60–150 min of autolysis ($p<0.05$). The increase in TBARS value of lipids from hepatopancreas indicated the formation of secondary lipid oxidation products (Chaijan *et al.*, 2006). The decomposition of hydroperoxides increased as the temperature increased (Shahidi and Spurvey, 1996). The hydroperoxides are decomposed to produce off-flavor compounds and the oil quality decreases (Choe and Min, 2006). It was noted that lipids extracted from hepatopancreas without prior autolysis had a TBARS value of 61.0 mg/kg sample. The result revealed that autoxidation of fatty acids in hepatopancreas took place during handling, storage or extraction. Marine lipids have been known to contain a high content of PUFA, which are prone to oxidation (Tocher and Harvie, 1988). The enhanced oxidation was noticeable when hepatopancreas was subjected to autolysis at 60°C, especially with extended processing time. The result confirmed the role of high temperature in acceleration of lipid oxidation. Further to this, mackerel liver oil storage at 40°C underwent oxidation more rapidly than that kept at 5°C during storage of 18 days (Sang and Jin, 2004). Thus, the addition of selected antioxidants in the hepatopancreas prior to autolysis or extraction could be a promising means to lower the oxidation in lipids.

4.5.5.3 *ρ*-anisidine value (AnV)

AnV of lipids from hepatopancreas increased as the autolysis times increased, especially after 60 min. (p<0.05) (Figure 9C). No changes in AnV were observed between the autolysis time of 30 and 60 min (p>0.05). In general, the similar result was observed in comparison with TBARS values. The *p*-anisidine reagent reacts with oxidation products, such as aldehydes (principally 2-alkenals and 2,4-dienals), producing a yellowish product. An increased AnV indicates an increase in the amount of the non-volatile oxidation product (Choe and Min, 2006). Typically, AnV increases as aldehydes are produced and then will decrease when the aldehydes reach a certain level (Gulla and Waghray, 2011). The AnV result reconfirmed the generation of non-volatile secondary product oxidation products of lipids during autolysis.

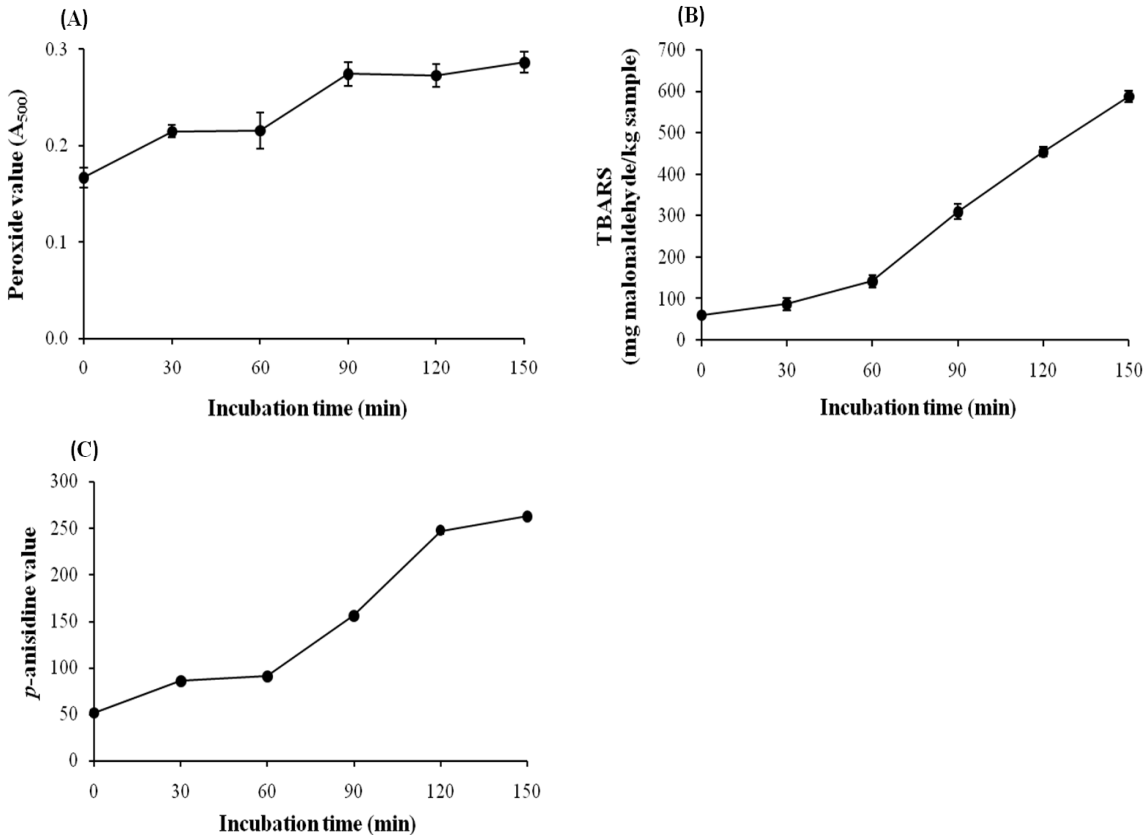

Figure 9 Changes in peroxide values (A), TBARS values (B) and *p*-anisidine values (C) of lipid from hepatopancreas of Pacific white shrimp incubated at 60°C for different times. Bars indicate standard deviations (n=3).

4.5.6 Free fatty acid content

Free fatty acid (FFA) contents of lipids extracted from hepatopancreas subjected to autolysis at 60°C for different times are shown in Figure 10. No change in FFA content in lipids was found during the first 60 min of autolysis (p>0.05). After 60 min, a marked increase in FFA content was noticeable in the lipids obtained (p<0.05). A slight increase in FFA contents was observed during 90–150 min of autolysis (p<0.05). Free fatty acids formed were likely prone to oxidation. This was coincidental with the increased lipid oxidation as indicated by the increase in PV, TBARS and AnV. Autoxidation of oil is accelerated by the presence of free fatty acids, mono- and di-acylglycerols (Choe and Min, 2006). Generally, lipids can undergo hydrolysis in the presence of moisture and heat (Chantachum *et al.*, 2000). Lipases, phospholipase A and phospholipase B are important enzymes involved in hydrolysis of lipids (Hwang and Regenstein, 1996). Hepatopancreas is the major source of lipase, which is able to hydrolyse the ester bond of triglyceride (Phillips *et al.*, 1984). The optimal temperature for lipase from hepatopancreas of squid (*Todarodes pacificus*) was 35–40°C (Park *et al.*, 2008). Therefore, endogenous lipase or phospholipase might be involved in hydrolysis of triglyceride or phospholipids, particularly during autolysis at 60°C.

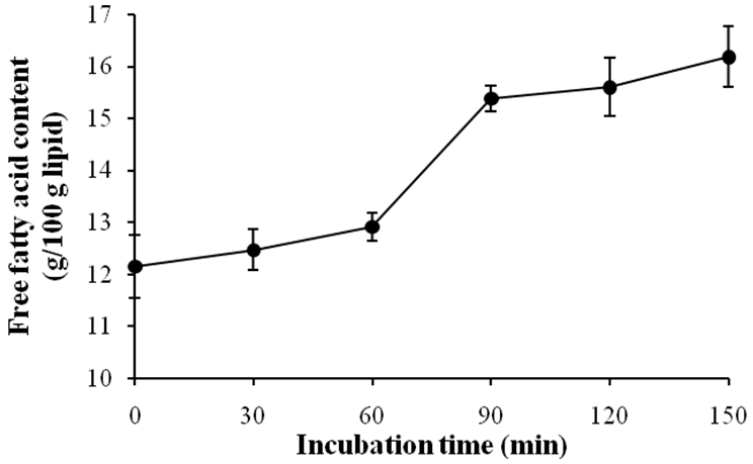

Figure 10 Changes in free fatty acid content of lipid from hepatopancreas of Pacific white shrimp incubated at 60°C for different times. Bars indicate standard deviations from triplicate determinations (n=3).

4.5.7 Changes in lipid composition

4.5.7.1 Lipid classes

Compositions of lipids from hepatopancreas of Pacific white shrimp without and with prior autolysis for 60 min are shown in Table 15. Phospholipids (PL) were found as the most predominant constituents (63.85–68.27%). Lipids contained free fatty acids (FFA) ranging from 16.90% to 20.82%. Small amounts of triglyceride (TG), diglyceride (DG) and monoglyceride (MG) were found. The result indicated that some triglyceride or phospholipids might be hydrolyzed to some degree, especially during autolysis. This was reflected by the non-significantly higher FFA content in lipids extracted from hepatopancreas with prior autolysis. Lipids from hepatopancreas without prior autolysis contained 4.35% TG, 16.90% FFA, 2.12% DG, 3.89% MG and 68.27% PL. Lipids from hepatopancreas with prior autolysis of 60 min consisted of 4.82% TG, 20.82% FFA, 2.64% DG, 7.88% MG and 63.85% PL. Generally, there was no difference in lipid composition between lipids extracted from hepatopancreas without and with prior autolysis, except for MG content, which was higher in lipid from hepatopancreas with prior autolysis (p<0.05). Lipid composition of krill from the Scotia Sea and krill from the Gerlache Strait were similar. The main lipids were phosphatidylcholine (33–36%), phosphatidylethanolamine (5–6%), triglyceride (33–40%), free fatty acids (8–16%) and sterols (1.4–1.7%) (Fricke *et al.*, 1984). Due to the high content of free fatty acids, the lipids could be susceptible to oxidation and the further refining should be implemented. Alternatively, the appropriate antioxidants can be added to prevent the oxidation.

Table 15 Composition of lipids from hepatopancreas of Pacific white shrimp without and with prior autolysis

Lipid sample	Compositions (% of total lipid content)				
	TG	**FFA**	**DG**	**MG**	**PL**
Without prior autolysis	4.35±0.96a	16.90±4.61a	2.12±0.59a	3.89±1.00a	68.27±7.16a
With prior autolysis	4.82±0.12a	20.82±1.51a	2.64±0.43a	7.88±0.40b	63.85±2.09a

Values are given as means ± SD from triplicate determinations.
Different letters in the same column indicate significant differences (p<0.05).

4.5.7.2 Fatty acid profiles

Fatty acid profiles of lipid from hepatopancreas of Pacific white shrimp without and with autolysis for 60 min are shown in Table 16. Lipids from hepatopancreas without prior autolysis contained 30.98% SAT, 26.40% MUFA and 38.11% PUFA. Lipids from hepatopancreas with prior autolysis for 60 min consisted of 31.38% SAT, 26.23% MUFA and 38.07% PUFA. Therefore, there was no difference in fatty acid profiles between lipids extracted from hepatopancreas without and with prior autolysis. Lipids extracted contained DHA as the most abundant PUFA, followed by EPA. In general, no differences in PUFAs were observed between lipids extracted from hepatopancreas without and with prior autolysis. Lipids extracted from hepatopancreas without autolysis had DHA and EPA of 10.45 and 3.31 g/100 g oil, respectively, whereas DHA and EPA at levels of 10.42 and 3.34 g/100 g oil, respectively, were found in lipids extracted from hepatopancreas with prior autolysis. Chaijan et al. (2006) reported that DHA is usually more abundant than EPA (up to 2–3 times) in marine lipids. Krill oil, extracted from Antarctic krill, represents a novel source of PUFA. Oil from custacean, including krill, contains a high amount of phospholipids rich in long chain *n*-3 PUFA, such as EPA and DHA (Bunea *et al.*, 2004). The amount of *n*-3 PUFAs of whole krill accounted for 19.0 ± 1.7% of fatty acid (Bustos *et al.*, 2003). It has been reported that environmental and physiological factors affect the level of PUFA in marine lipids. Additionally, the extraction methods and solvents used also had the impact on fatty acid composition in krill oil (Lambertsen and Braekkan, 1971). The result revealed that lipids from hepatopancreas of Pacific white shrimp were an excellent source of *n*- 3 fatty acids, which can be of health benefit.

Table 16 Fatty acid profile of lipids from hepatopancreas of Pacific white shrimp without and with prior autolysis

Fatty acids (g/100 g oil)	**Without prior autolysis**	**With prior autolysis**
C14:0	1.26	1.28
C14:1	0.23	0.23
C15:0	0.78	0.80
C15:1	0.35	0.36
C16:0	23.40	23.72
C16:1 *n*-7	2.72	2.77
C17:0	0.96	0.98
C17:1	0.47	0.48
C18:0	4.02	4.07
C18:1 *n*-9	20.08	20.18
C18:2 *n*-6	17.88	17.97
C18:3 *n*-3	1.18	1.17
C20:0	0.27	0.26
C20:1 *n*-9	1.70	1.39
C20:2 *n*-6	1.57	1.56
C20:3 *n*-6	0.17	0.13
C20:3 *n*-3	0.25	0.25
C20:4 *n*-6	1.46	1.47
C20:4 *n*-3	0.18	0.18
C20:5 *n*-3 (EPA)	3.31	3.34
C21:0	0.15	0.14
C22:1 *n*-9	0.21	0.20
C22:1 *n*-11, *n*-13	0.64	0.62
C22:4 *n*-6	0.22	0.21
C22:5 *n*-3	0.82	0.78
C22:5 *n*-6	0.62	0.59
C22:6 *n*-3 (DHA)	10.45	10.42
C23:0	0.14	0.13
Saturated fatty acid (SFA)	30.98	31.38
Monounsaturated fatty acid (MUFA	26.40	26.23
Polyunsaturated fatty acid (PUFA)	38.11	38.07

4.6 Conclusion

Prior autolysis of hepatopancreas of Pacific white shrimp at 60 C for 60 min increased the extraction yield without any negative effects on fatty acid composition. However, hydrolysis and oxidation took place to some extent. Lipids had high contents of DHA, EPA as well as astaxanthin. Thus, lipids from hepatopancreas can serve as a rich source of valuable nutrients for human consumption, especially when lipid extraction without hazardous chemicals is implemented.

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CHAPTER 5

Antioxidative activities of hydrolysates from seabass skin prepared using protease from hepatopancreas of Pacific white shrimp

5.1 Abstract

Antioxidative activities of hydrolysates from skin of seabass (*Lates calcarifer*) with different degrees of hydrolysis (DH: 10-40%) prepared using an ammonium sulfate fraction (ASF) from Pacific white shrimp hepatopancreas and commercial Alcalase were compared. The hydrolysate prepared using ASF or Alcalase had increases in 2,2-diphenyl-1-picrylhydrazyl (DPPH), 2,2-azino-bis (3-ethylbenzothiazoline-6-sulfonic acid) (ABTS) radical scavenging activities and ferric reducing antioxidative power (FRAP) as DH increased (p<0.05). When the hydrolysate prepared using ASF with 40%DH was subjected to a gastrointestinal model system (GIMs), ABTS radical scavenging activity and chelating activity increased, especially in the duodenal condition. The hydrolysate (500-2000 mg/L) could inhibit lipid oxidation in a lecithin liposome system in a dose dependent manner. Based on gel filtration using a Sephadex G^{TM}-15 column, peptide with a molecular weight of 364 Da showed the strongest ABTS radical scavenging activity. Therefore, the extract from hepatopancreas could be used for increasing of DH from seabass skin hydrolysates.

5.2 Introduction

Pacific white shrimp and its products have become economically important for Thailand. By the year 2010, the total amount of frozen Pacific white shrimp and products was 408,000 metric tonnes and the products were mainly exported to the USA and Japan (Lebel *et al.*, 2010). During shrimp processing, a large amount of by-products including cephalothorax, shell, etc. are generated (Binsan *et al.*, 2008). Amongst all shrimp products, whole shrimp without hepatopancreas are currently showing increased demand. The hepatopancreas is removed using a vacuum sucking machine (Takeungwongtrakul and Benjakul, 2014). Shrimp hepatopancreas

can be a major source of proteases, especially trypsin and chymotrypsin (Sriket *et al.*, 2012). Proteases in the hepatopancreas from freshwater prawn actively hydrolyzed various proteinaceous substrates (Sriket *et al.*, 2012).

Seabass (*Lates calcarifer*) are very popular in South-East Asia owing to their white flesh and delicacy (Sinthusamran *et al.*, 2013). During fillet production, skin is generated and can serve as a potential source for gelatin (Sinthusamran *et al.*, 2013). Additionally, gelatin from fish skin has been used for production of gelatin hydrolysate with bioactivities (Ngo *et al.*, 2010). The beneficial effects of bioactive peptides are well known in scavenging free radicals and reactive oxygen species or in preventing the oxidative damage by interrupting the radical chain reaction of lipid peroxidation (Harnedy and FitzGerald, 2012; Kim and Wijesekara, 2010). Gelatin hydrolysate from bigeye snapper skin, possessing antioxidative activity including DPPH and ABTS radicals scavenging activity and ferric reducing antioxidant power, could be prepared with the aid of protease from fish pyloric caeca (Khantaphant and Benjakul, 2008; Phanturat *et al.*, 2010). To reduce the cost of commercial protease used for gelatin hydrolysate production, the cheap proteases from shrimp processing byproducts, especially from hepatopancreas, could be an alternative. The direct hydrolysis of pretreated skin, a major source of collagen and gelatin, without prior gelatin extraction, can shorten the processing time and lower the operation cost. Furthermore, new peptides could be generated due to varying specificity towards substrates amongst different proteases. Those peptides can serve as the functional supplement in foods or drinks. Therefore, the present study aimed to investigate the impact of protease from hepatopancreas in comparison with commercial protease on hydrolysis of skin of seabass and to study the antioxidative activities and characteristics of the resulting hydrolysate.

5.3 Objective

To prepare gelatin hydrolysates from seabass skin with antioxidative activity using the protease from Pacific white shrimp hepatopancrease.

5.4 Materials and methods

5.4.1 Chemicals

Alcalase was obtained from Novozyme (Bagsvaerd, Denmark). The compounds 2,4,6-trinitrobenzenesulfonic acid (TNBS), 2,2′-azinobis (3-thylbenzothiazoline-6-sulfonic acid) (ABTS), 2,2-diphenyl-1-picrylhydrazyl (DPPH), 2, 4, 6-tripyridyltriazine (TPTZ), 3-(2-pyridyl)-5,6-diphenyl-1,2,4-triazine-4´,4´´-disulfonic acid sodium salt (ferrozine), ethylenediaminetetraacetic acid (EDTA), pepsin from porcine gastric mucosa (EC 3.4.23.1), pancreatin from porcine pancreas, trypsin from bovine pancreas (EC 3.4.21.4) and 6-hydroxy- 2,5,7,8-tetramethyl-chroman-2-carboxylic acid (Trolox) were procured from Sigma-Aldrich, Inc. (St. Louis,MO., U.S.A.). Sephadex G-15 and gel filtration calibration kit (vitamin B12, flavin adenine dinucleotide and glycine-tryrosine) were obtained from GE Healthcare (Uppsala, Sweden).

5.4.2 Preparation of ammonium sulfate fraction from hepatopancreas

Hepatopancreas of Pacific white shrimp was collected from Sea wealth frozen food Co., Ltd., Songkhla province, Thailand. Hepatopancreas was packaged in polyethylene bag, stored in ice using a sample/ice ratio of 1:2 (w/w) and transported to the Department of Food Technology, Prince of Songkla University within 2 h. Upon arrival, hepatopancreas was powdered in liquid nitrogen and homogenized in three volumes of acetone at -20°C for 30 min according to the method of Kishimura and Hayashi (2002). The homogenate was filtered in vacuo on Whatman No. 4 filter paper (Whatman International Ltd., Maidstone, UK). The residue obtained was then homogenized in two volumes of acetone at -20°C for 30 min, and then the residue was left at room temperature until dried and free of acetone odor.

To prepare the crude extract, acetone powder was suspended in 10 mM Tris–HCl, pH 8.0 containing 1 mM $CaCl_2$ (an extraction buffer) at a ratio of 1:50 (w/v) and stirred continuously using a magnetic stirrer model BIG SQUID (IKA®-Werke GmbH & CO.KG, Staufen, Germany) at 4°C for 3 h. The suspension was centrifuged for 10 min at 4°C at 10,000×g to remove the tissue debris using a

refrigerated centrifuge (Beckman Coulter, Avanti J-E Centrifuge, Fullerton, CA, USA). The solution was referred to as "crude extract".

Crude extract was subjected to ammonium sulfate precipitation at 40-60% saturation as described by Khantaphant and Benjakul (2008). After the addition of ammonium sulfate, the mixture was stirred gradually at 4°C for 30 min. Thereafter, the mixture was centrifuged at 8000×g for 30 min at 4°C and the pellet obtained was dissolved in the minimum volume of 50 mM Tris–HCl buffer, pH 8.0. The solution was dialysed against 20 volumes of the extraction buffer overnight at 4°C with three changes. The dialysate was kept in ice and referred to as 'ammonium sulfate fraction, ASF'. The supernatant was lyophilised using a SCANVAC CoolSafe™ freeze-dryer (CoolSafe 55, ScanLaf A/S, Lynge, Denmark). The lyophilised sample with proteolytic activity of 340 unit/g powder (10 g) was dissolved with 50 mL of cold distilled water (4°C). The solution was determined for protease activity and further used.

5.4.3 Protease activity assay

Both ASF and Alcalase were determined for protease activity using casein as substrate as per the method of An *et al.* (1994). The assay was performed at pH 8 and 60°C. One unit of activity was defined as that releasing 1 μmol of tyrosine per min (μmol Tyr/min).

5.4.4 Preparation of hydrolysate from skins of seabass

Firstly, skins (1x1 cm^2) were soaked in 0.1 M NaOH with a skin/solution ratio of 1:10 (w/v) at room temperature (25°C) with a gentle stirring using an overhead stirrer equipped with a propeller (RW 20.n, IKA Labortechnik, Staufen, Germany) at a speed of 150 rpm. The solution was changed every 30 min for totally three times to remove non-collagenous proteins and pigments. Alkaline-treated skins were washed with tap water until the neutral or faintly basic pH wash water was obtained. The skins were then soaked in 0.05 M acetic acid with a skin/solution ratio of 1:10 (w/v) for 2 h at room temperature with a gentle stirring to swell the collagenous material in fish skin. Acid-treated skins were washed as previously

described. Swollen skins were added with distilled water at a ratio of 1:10 (w/v). The pH of the mixture was adjusted to pH 8.0 using 2 M NaOH. The mixture was incubated at 60°C for 15 min.

To compare hydrolysis efficacy towards swollen skins, ASF or Alcalase was added into pre-incubated mixture at levels of 5 and 10 units /g swollen skin dry matter. At hydrolysis time designated (5, 10, 15, 20, 30, 40, 60, 90, 120 and 180 min), 1 mL of sample was taken and mixed with 1 mL of 2% SDS solution (90°C) before placing in a temperature controlled water bath (Memmert, Bavaria, Germany) at 90°C for 10 minto inactivate the enzyme and solubilize proteins. The sample was used for DH determination. The hydrolysis time rendering the initial velocity was selected for further study.

To prepare hydrolysates with different DH (10, 20, 30 and 40%), ASF or Alcalase with different amounts were added as described by Benjakul and Morrissey (1997). The hydrolysis was carried out for 60 min at 60 °C, prior to termination as previously described. The obtained hydrolysates were centrifuged at 8000×g for 10 min. The supernatants were lyophilized and further subjected to analyses.

5.4.5 Analyses

5.4.5 1 Determination of DH

DH of hydrolysate was determined according to the method of Benjakul and Morrissey (1997). Hydrolysate samples with the 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 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 using a UV-1601 spectrophotometer (Shimadzu, Kyoto, Japan) and α-amino group was expressed in terms of *L*-leucine. The DH was defined as follows:

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

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

5.4.5 2 Determination of antioxidative activities

The hydrolysates with different DHs (10, 20, 30 and 40%) were determined for antioxidative activities as follows:

5.4.5.2.1 DPPH radical scavenging activity

DPPH radical scavenging activity was determined as described by Wu, Khantaphant and Benjakul (2008) with a slight modification. Freeze-dried hydrolysate sample (5 mg/mL) was dissolved in distilled water. Sample (1.5 mL) was added with 1.5 mL of 0.15 mM DPPH in 95% methanol. 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 spectrophotometer (UV-1800, 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)/g sample.

5.4.5.2.2 ABTS radical scavenging activity

ABTS radical scavenging activity was determined as described by Binsan *et al.* (2008). The stock solutions included 7.4 mM ABTS solution and 2.6 mM potassium persulfate solution. The working solution was prepared by mixing two stock solutions in equal quantities and 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 A_{734} of 1.1 ± 0.02 using a spectrophotometer. Fresh ABTS solution was prepared daily. Freeze-dried hydrolysate sample (1 mg/mL) was dissolved in distilled water. Sample (150 μL) 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)/g sample.

5.4.5 2.3 Ferric reducing antioxidant power (FRAP)

FRAP was assayed according to Benzie and Strain (1996). Stock solutions included 300 mM acetate buffer (pH 3.6), 10 mM TPTZ (2,4,6-tripyridyl-*s*-triazine) solution in 40 mM HCl, and 20 mM $FeCl_3 \cdot 6H_2O$ solution. A working solution was prepared freshly by mixing 25 mL of acetate buffer, 2.5 mL of TPTZ solution and 2.5 mL of $FeCl_3 \cdot 6H_2O$ solution. The mixed solution was incubated at 37°C for 30 min and was referred to as FRAP solution. Freeze-dried hydrolysate sample (10 mg/ml) was dissolved in distilled water. Sample (150 µL) was mixed with 2850 µL of FRAP solution and kept for 30 min in the dark. The ferrous tripyridyltriazine complex (colored product) was measured by reading the absorbance at 593 nm. The standard curve was prepared using Trolox ranging from 50 to 600 µM. The activity was expressed as µmol Trolox equivalents (TE)/g sample.

5.4.5 2.4 Chelating activity towards Fe^{2+}

Chelating activity towards Fe^{2+} was measured as according to Thiansilakul *et al.* (2007). Freeze-dried hydrolysate sample (10 mg/ml) was dissolved in distilled water. The sample was mixed with 0.1 mL of 2 M $FeCl_2$ and 0.2 mL of 5 M ferrozine. The reaction mixture was allowed to stand for 20 min at room temperature (26–28°C). The absorbance was then read at 562 nm using a spectrophotometer. The blank was prepared in the same manner except that distilled water was used instead of the sample. The Fe^{2+} chelating activity was expressed as EDTA equivalents (EE)/g sample. A standard curve of 0–50 µM EDTA was prepared.

The hydrolysate prepared using ASF with the DH showing the highest antioxidative activity was selected for further study.

5.4.6 Antioxidative activity of the selected skin hydrolysate in lecithin liposome model system

Antioxidative activity of the hydrolysate with the selected DH in a lecithin liposome model system was determined according to the method of Phanturat *et al.* (2010). Lecithin liposome system was prepared by suspending lecithin in deionised water at a concentration of 8 mg/mL. The mixture was stirred with a glass rod, followed by sonification for 30 min in a sonicating bath (Elma Model S30H, Singen, Germany). The hydrolysate (3 mL) was added to the lecithin liposome system (15 mL) to obtain a final concentration of 500, 1000 and 2000 mg/L. The mixture was sonicated for 2 min. To initiate the reaction, 20 mL of 0.15 M cupric acetate were added. The mixture was shaken in the dark at 120 rpm using a shaker (Heidolph Model Unimax 1010, Schwabach, Germany) at 37°C. The system containing 100 mg/L Trolox was also prepared. The control was prepared in the same manner, except that distilled water was used instead of sample or Trolox. Oxidation in lecithin liposome system was monitored during 60 h of incubation by determining the formation of TBARS and conjugated dienes.

5.4.6.1 Determination of thiobarbituric acid reactive substances (TBARS)

TBARS were determined as described by Buege and Aust (1978) with a slight modification. The sample (0.5 mL) was homogenized with 2.5 mL of TBARS solution (0.375% TBA, 15% TCA and 0.25 M HCl). The mixture was heated in boiling water for 10 min to develop the pink color. Then the mixture was cooled with running water and centrifuged at 5000×g for 10 min at room temperature using Hettich centrifuge (Hettich Model MIKRO-20, Tuttlingen, Germany). The supernatant was collected and measured at 532 nm using a spectrophotometer. TBARS values were calculated from a standard curve of malondialdehyde (MDA) (0–10 mg/L) precursor and expressed as mg MDA eqivalent/mL liposome.

5.4.6.2 Determination of conjugated diene

Conjugated dienes formed in the sample was measured according to the method of Frankel *et al.* (1997). The sample (0.1 mL) was dissolved in methanol (5.0 mL) and the absorbance at 234 was read. The content of conjugated dienes was expressed as the increase in absorbance at 234 nm.

5.4.7 Stability in gastrointestinal tract model system

Gastrointestinal tract model system was prepared according to the method of Lo *et al.* (2006) with a slight modification. The hydrolysate was dissolved in distilled water to obtain a concentration of 0.5 g/mL. The solution was adjusted to pH 2.0 with 1 M HCl and pepsin dissolved in 0.1 M HCl was added to obtain the final concentration of 40 g pepsin /kg hydrolysate. The mixture was incubated at 37°C for 1 h with a continuous shaking (Memmert Model SV 1422, Schwabach, Germany). Thereafter, the pH of the reaction mixture was raised to 5.3 with 1 M NaOH before the addition of 20 g pancreatin/ kg hydrolysate. Subsequently, the pH of mixture was adjusted to 7.5 with 1 M NaOH. The mixture was incubated at 37°C for 3 h with a continuous shaking. The digestion was terminated by submerging the mixture in boiling water for 10 min. During digestion, the mixture was randomly taken at 0, 20, 40, 60, 80, 100, 120, 150, 180, 210 and 240 min for determination of ABTS radical scavenging activity and chelating activity.

5.4.8 Fractionation of antioxidative peptides from skin hydrolysate

The hydrolysate was fractionated using Sephadex^{TM} G15 column (2.5×50 cm). The sample (100 mg) was dissolved in distilled water (2 mL). The mixture was loaded onto a column and the elution was performed using distilled water at a flow rate of 0.5 mL/min. The 3 mL-fractions were collected and their absorbance was monitored at 220 nm. The standards, including vitamin B12 (1,355 Da), flavin adenine dinucleotide (829.5 Da) and Gly-Tyr (238.25 Da) were used. Blue dextran (2,000 kDa) was used to measure the void volume of the column. The fractions were determined for their ABTS radical scavenging activity. The molecular weight (MW) of the fraction with ABTS radical scavenging activity was calculated as described

earlier. Protein content of all fractions was determined using the method of Lowry *et al.* (1951).

5.4.9 Statistical analysis

Experiments were run in triplicate using three different lots of samples. Data were subjected to analysis of variance (ANOVA). Comparison of means was carried out by Duncan's multiple range test (Steel and Torrie, 1980). Statistical analysis was performed using the Statistical Package for Social Science (SPSS 11.0 for windows, SPSS Inc., Chicago, IL, USA).

5.5 Results and discussion

5.5.1 Hydrolysis of swollen seabass skin using ASF and Alcalase

When swollen seabass skin was hydrolyzed using ASF and Alcalase at levels of 5 and 10 units/g swollen skin, different degrees of hydrolysis (DH) were noticeable as a function of time, depending on the type of enzyme and enzyme levels. Generally, hydrolysis rate was high within the first 30 min, followed by a slower hydrolysis rate (Figure 11A). The rapid hydrolysis in the initial phase indicated that a large number of peptide bonds were hydrolyzed (Shahidi *et al.*, 1995). The hydrolysis rate was decreased, mainly due to a decrease in the available hydrolysis sites, enzyme autodigestion and/or product inhibition (Khantaphant and Benjakul, 2008). At the same level of enzyme and hydrolysis time, higher DH was observed when ASF was used, in comparison with Alcalase. This suggested that swollen skin was more hydrolyzed by AFS from Pacific white shrimp hepatopancreas than with Alcalase.

DH has been used as an indicator for the cleavage of peptide bond and it can be maneuvered during hydrolysis to obtain the desirable properties of bioactive peptides (Amiza *et al.*, 2012). The biological properties of protein hydrolysates depend on the protein substrate, the specificity of the enzyme used for proteolysis, the conditions used during hydrolysis and the DH (Bougatef *et al.*, 2009). Several properties of protein hydrolysates are closely related to the DH, which is associated with chain length of peptides (Khantaphant and Benjakul, 2008). During incubation at 60°C used for hydrolysis, collagens in swollen skin underwent thermal denaturation,

in which amorphous gelatin was formed. Sinthusamran *et al.* (2013) reported that collagen from skin of seabass had the T_{max} of 33.33°C. Amorphous α- or β-chains might have more cleavage sites towards enzymes. It was noted that proteases from Pacific white shrimp hepatopancrease were able to cleave gelatin in the skin effectively as evidenced by high DH.

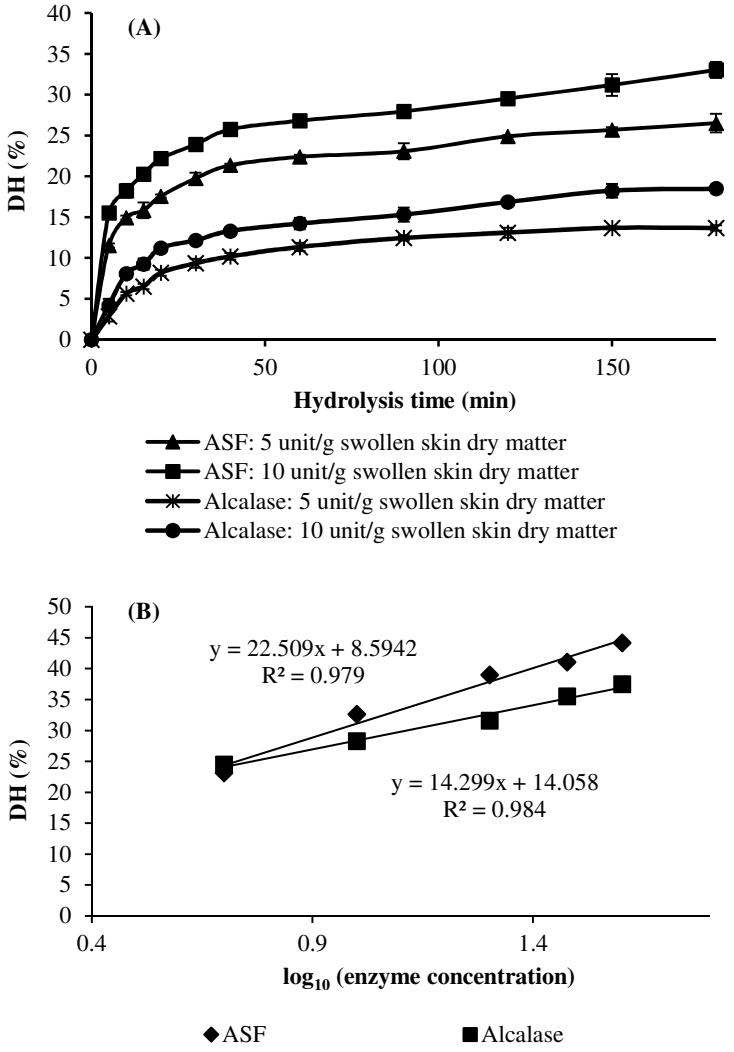

Figure 11 Degree of hydrolysis (DH) of gelatin from swollen seabass skin during hydrolysis with ASF of hepatopancreas from Pacific white shrimp or Alcalase at different concentrations (A) and relationship between DH and log enzyme concentration (unit/g swollen skin dry matter) of ASF or Alcalase for hydrolysis of swollen skin (B). Bars represent the standard deviation (n=3).

When $\log_{10}$ (enzyme unit) was plotted against DH after hydrolysis for 60 min, a linear relationship was obtained (Figure 11B). Relationship between %DH and log of subtraction of Alcalase and Flavozyme was reported by Klompong *et al.* (2007) when yellow stripe trevally muscle protein was used as a substrate. At the same enzyme concentration, the hydrolysate prepared using ASF showed higher DH than that treated with Alcalase. This reconfirmed the higher ability of ASF in hydrolyzing proteins, mainly gelatin from swollen skins, compared with Alcalase (Figure 11A). Several parameters, such as substrate, enzyme-substrate ratio, temperature and time involved in enzymatic hydrolysis generally influenced the DH of the resulting hydrolysate (Kristinsson and Rasco, 2000). From this relationship, the amount of enzyme required for skin hydrolysis to obtain the required DH (10, 20, 30 and 40%) was calculated.

5.5.2 Antioxidative activities of skinhydrolysates with various DH produced by ASF and Alcalase

5.5.2.1 DPPH radical scavenging activity

DPPH radical scavenging activities of hydrolysates prepared using ASF and Alcalase with different DH (10-40%) are depicted in Figure 12A. Hydrolysates produced by ASF exhibited increased DPPH radical scavenging activity as DH increased ($p<0.05$). However, there was no difference in DPPH radical scavenging activity between hydrolysate prepared by using Alcalase with DH of 10, 20 and 30% ($p>0.05$). The sharp increase in DPPH radical scavenging activity was noticeable for hydrolysates prepared by both proteases with 40%DH. The results indicated that antioxidative peptides were produced during the hydrolysis, especially with increasing cleavage of peptide bonds. At 40% DH, hydrolysate prepared using Alcalase showed slightly higher activity than that prepared using AFS ($p<0.05$). Kittiphattanabawon *et al.* (2012) reported that DPPH radical scavenging activity of blacktip shark skin prepared using papaya latex enzyme increased with increasing DH up to 40% ($p<0.05$). DPPH radical scavenging activity of peanut protein hydrolysate increased as DH increased from 10 to 20% but no difference in activity was observed as DH increased from 20 to 40%. DPPH is a stable free radical that shows maximal

absorbance at 517 nm in ethanol. When DPPH encounters a hydrogen atom-donating substance, such as an antioxidant, the radical is scavenged. The color is changed from purple to yellow and the absorbance is reduced (Kittiphattanabawon *et al.*, 2012). Therefore, skin hydrolysate obtained could donate hydrogen atom to free radicals and become more stable diamagnetic molecule, leading to the termination of the radical chain reaction (Binsan *et al.*, 2008). Nevertheless, the efficiency in hydrogen donation of peptides produced was governed by enzymes used for hydrolysis.

5.5.2.2 ABTS radical scavenging activity

ABTS radical scavenging activities of skin hydrolysates prepared using ASF and Alcalase with different DH are shown in Figure 12B. With increasing DH up to 30%, the hydrolysates prepared using ASF and Alcalase showed increases in ABTS radical scavenging activity ($p<0.05$). Nevertheless, no differences in activity were observed between hydrolysate prepared using ASF with 10 and 20% DH ($p>0.05$). It was noted that similar ABTS radical scavenging activity was found between hydrolysates with 30 and 40% DH, regardless of enzymes used. ABTS radical scavenging activity is based on the ability of antioxidants to donate a hydrogen atom or an electron to stabilize radicals, by converting it to the non-radical species (Binsan *et al.*, 2008). The pre-formed radical monocation of $ABTS^{\cdot+}$ is generated by oxidation of ABTS with potassium persulfate and is reduced in the presence of hydrogen-donating antioxidants and of chain breaking antioxidants (Binsan *et al.*, 2008). The ABTS radical scavenging activity assay can be applied to both lipophilic and hydrophilic compounds, and has been widely used as an antioxidant activity assay (Re *et al.*, 1999). Gelatin hydrolysates from bigeye snapper skin with DH ranging from 5 to 25% prepared using Alcalase had the increased ABTS scavenging activity with increasing DH (Phanturat *et al.*, 2010). The results suggest that skin hydrolysates, especially at 30 or 40% DH, have the ability to scavenge free radicals, thereby preventing lipid oxidation via a chain breaking reaction.

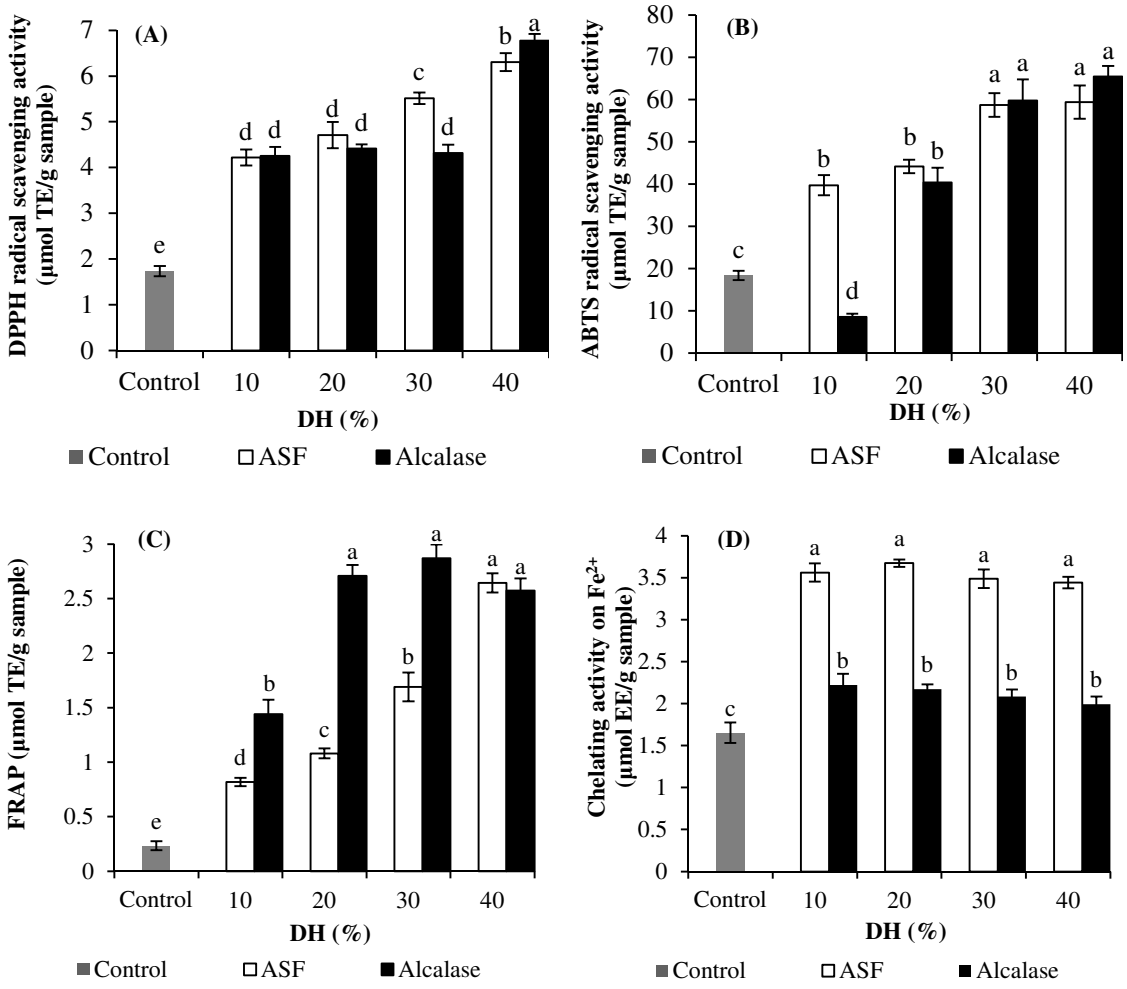

Figure 12 DPPH radical scavenging activity (A), ABTS radical scavenging activity (B), FRAP (C) and metal chelating activity (D) of hydrolysates from swollen seabass skin prepared using ASF of hepatopancrease from Pacific white shrimp with different degrees of hydrolysis (DH). Bars represent the standard deviation (n=3). Different letters within the same parameter indicate the significant differences (p<0.05).

5.5.2.3 Ferric reducing antioxidant power (FRAP)

Skin hydrolysates prepared using ASF and Alcalase had increases in FRAP when DH increased (p<0.05) (Figure 12C). At the same DH, hydrolysates prepared using Alcalase showed a higher activity than those produced using ASF (p<0.05), except at 40% DH, in which both hydrolysates showed similar FRAP

(p>0.05). There were no differences in FRAP between hydrolysates prepared using Alcalase with DH of 20-40% (p>0.05). The greater reducing power indicated that hydrolysates could donate an electron to free radicals, leading to the prevention or retardation of propagation (Klompong *et al.*, 2007). FRAP is generally used to measure the capacity of a substance in reducing TPTZ-Fe(III) complex to TPTZ-Fe(II) complex (Binsan *et al.*, 2008). Therefore, the hydrolysis by ASF most likely increased FRAP of the resulting hydrolysate via the enhancement of reducing power towards free radicals. As a result, propagation step could be terminated in the presence of hydrolysate with appropriate DH.

5.5.2.4 Metal-chelating activity

Metal-chelating activity of skin hydrolysates prepared by both ASF and Alcalase (Figure 12D) was higher than the control (gelatin without hydrolysis) (p<0.05). When the same enzyme was used for hydrolysis, the resulting hydrolysates showed a similar chelating activity (p>0.05). At the same DH, hydrolysate prepared using ASF showed a higher chelating activity than that produced by Alcalase (p< 0.05). Metal chelating activity of hydrolysate from yellow stripe trevally muscle prepared by both Alcalase (HA) and Flavozyme (HF) increased with increasing DH. HF hydrolysate generally had a higher (p<0.05) chelating activity than did HA at the same DH tested (Klompong *et al.*, 2007). Therefore, peptides in hydrolysates could chelate the prooxidative metals, leading to the decreased lipid oxidation. However, peptides in hydrolysates more likely had different metal ion chelating capacity, depending on the amino acid sequences and chain length of peptide fragments. Transition metals, such as Fe, Cu and Co in foods affect both 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). Thus, skin hydrolysate could act as the secondary antioxidant, which could scavenge prooxidative metal ions. Skin hydrolysate prepared using AFS from Pacific white shrimp hepatopancres with 40%DH was further investigated for its preventive effect in a lecithin liposome as well as its stability in the gastrointestinal tract.

5.5.3 Antioxidative activity of skinhydrolysate in lecithin liposome model system

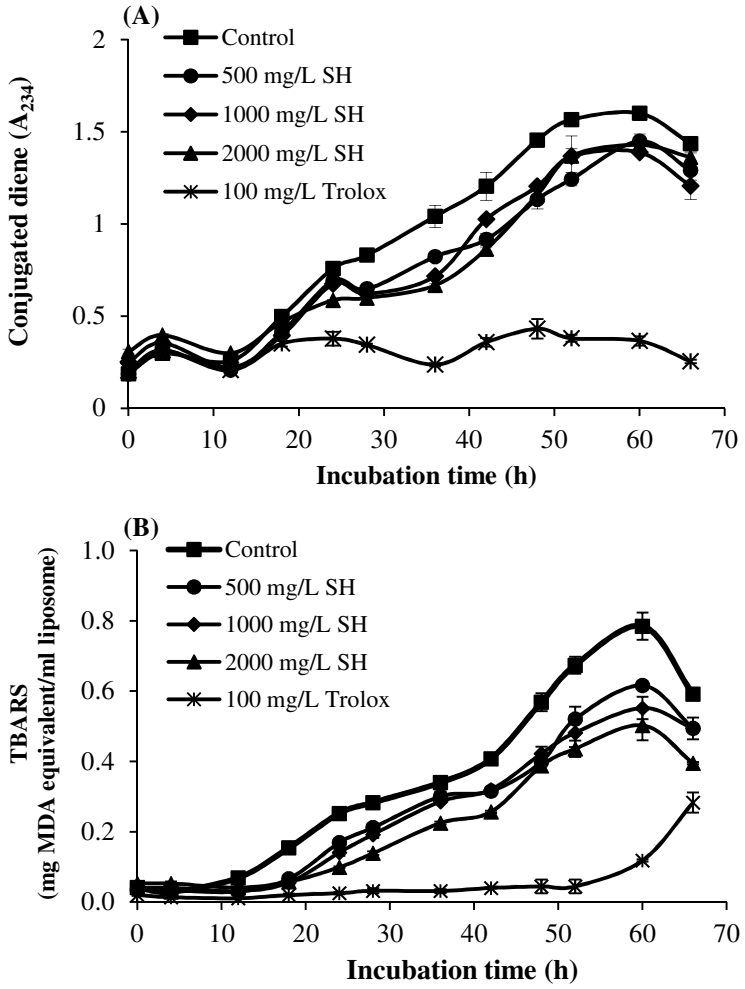

Figure 13 The formation of conjugated diene (A) and TBARS (B) in lecithin liposome system containing hydrolysate from swollen seabass skin prepared using ASF of hepatopancrease from Pacific white shrimp at different levels.SH denote skin hydrolysate. Bars represent the standard deviation *(n*=3).

Skin hydrolysate prepared using ASF of hepatopancrease from Pacific white shrimp with 40%DH at different levels affected the oxidation of the lecithin liposome system differently as indicated by varying conjugated dienes (CD) (Figure 13A) and TBARS values (Figure 13B). Generally, the oxidation of systems increased when the incubation time increased (p<0.05) up to 60 h. Thereafter, a slight decrease in CD was found. The formation of dienes occurs during the early stages of lipid

oxidation (Frankel *et al.*, 1997). CD and hydroperoxides are further decomposed to secondary products. The decrease or reaching a plateau of conjugated diene was generally accompanied by an increase in TBARS (Binsan *et al.*, 2008). Amongst all samples, the control (without hydrolysate or Trolox) had a higher CD than others ($p<0.05$). However, no marked changes in CD of liposome system added with 100 ppm trolox were observed throughout 66 h of incubation ($p>0.05$). No pronounced differences in CD were noticeable among systems added with hydrolysates with different levels ($p>0.05$) throughout the storage period.

All samples had the increase in TBARS up to 60 h ($p<0.05$). Subsequently, TBARS value was decreased to some degree ($p<0.05$).The rate of increase varied with the concentration of hydrolysate used. Systems containing hydrolysate at 2000 mg/L had lower TBARS than those added with hydrolysate at levels of 500 and 1000 mg/L ($p<0.05$).After the 60 h incubation, decreased TBARS was observed in all samples, suggesting the loss of oxidation products. The secondary products with low MW were possibly lost, leading to lower amounts of such products (Stahnke, 1995). It was noted that skin hydrolysate had the lower efficacy in prevention of oxidation in lecithin liposome system than trolox during the incubation of period 66 h.

5.5.4 Changes in antioxidative activity of skinhydrolysate in gastrointestinal tract model system (GIMs)

Antioxidative activity of skin hydrolysate prepared using ASF with 40%DH in GIMs was monitored by measurement of ABTS radical scavenging activity and chelating activity (Figure 14). Skin hydrolysates showed a slight increase in ABTS radical scavenging activity and chelating activity during pepsin digestion ($p<0.05$). With further hydrolysis in intestinal simulated system, sharp increases in ABTS radical scavenging were obtained within the first 20 min under duodenal conditions. No changes in ABTS radical scavenging activity were found between 80 and 180 min ($p>0.05$). Subsequently, a slight increase in ABTS radical scavenging activity was obtained. For metal chelating activity, the continuous increases were noticeable up to 210 min ($p<0.05$). No further change was found during 210 and 240 of incubation ($p>0.05$). Khantaphant *et al.* (2011) also found increased antioxidative

activity of protein hydrolysate from the muscle of brownstripe red snapper using Flavourzyme after being ingested in the simulated model system. The result suggested that pancreatin might cleave the peptides to some degrees, leading to the release of new antioxidative peptides. This could enhance the antioxidative activities of hydrolysates. Generally, gastrointestinal tract may actually leads the generation of more potent bioactive peptides (Megías et al., 2009). A number of studies have indicated that reactive oxygen species (ROS), generated in the digestion process, are prints of certain diseases (Srigiridhar *et al.*, 2001). The results showed that the antioxidative activity of skin hydrolysate could be increased in the gastrointestinal tract as mediated by its extent of enzymes.

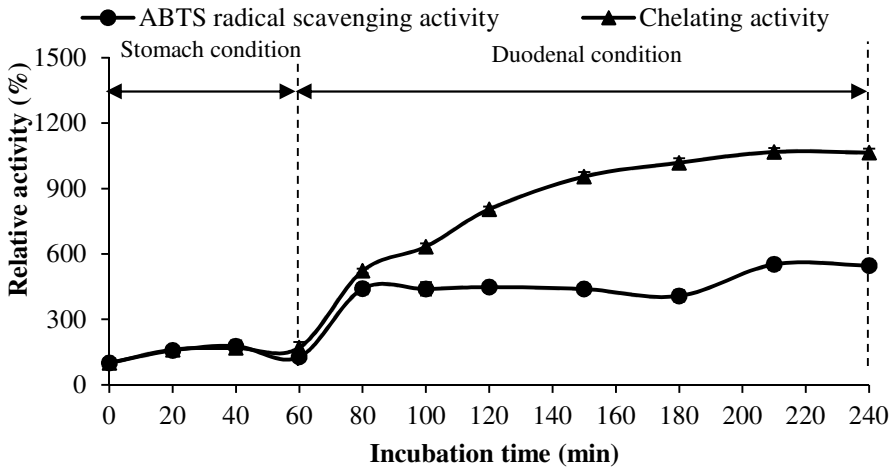

Figure 14 Antioxidative activities of hydrolysate from swollen seabass skin with 40% DH prepared using ASF of hepatopancrease from Pacific white shrimp in gastrointestinal tract model system. Bars represent the standard deviation (*n*=3).

5.5.5 Fractionation of antioxidative peptides in skin hydrolysates

Skin hydrolysate prepared using ASF of hepatopancrease with 40%DH was fractionated using Sephadex^{TM}-G15 gel filtration chromatography (Figure 15).The hydrolysate had three peaks of A_{220}, indicating the presence of peptides with varying MW in the hydrolysate. The fraction containing peptides with a MW of 364 Da showed the highest ABTS radical-scavenging activity. Peptide with MWs of 1505 and 156 Da also had ABTS radical scavenging activity. Kittiphattanabawon *et*

al. (2012) reported that gelatin hydrolysates from blacktip shark skin prepared using papaya latex enzyme (40% DH) contained antioxidative peptides with a MW of approximately 644 Da. Antioxidative peptides of gelatin hydrolysate from tilapia skin prepared using properase E and multifect neutral were purified using Sephadex G-15 and SP Sephadex C-25 gel chromatographies. The MW of the two peptides were 317.33 Da and 645.21 Da (Zhang *et al.*, 2012). The antioxidative peptide of gelatin hydrolysate from bigeye snapper skin prepared using Alcalase combined with pyloric caeca extract had a MW of 1.7 kDa (Phanturat *et al.*, 2010). The result suggested that skin hydrolysate most likely contained certain peptides with radical scavenging activity, which could terminate radical chain reaction. Further identification of antioxidative peptides is needed.

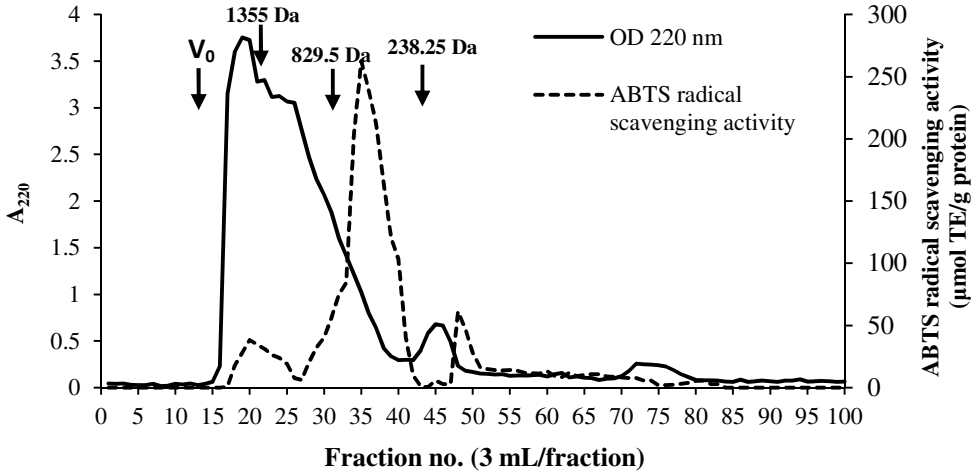

Figure 15 Separation of antioxidative peptides inhydrolysate from swollen seabass skinwith 40% DH prepared using ASF of hepatopancrease from Pacific white shrimp by Sephadex^{TM} G-15 column.

5.6 Conclusion

Proteases from hepatopancrease of Pacific white shrimp could hydrolysethe pretreated skin effectively. Hydrolysate exhibited various antioxidative activities, depending on the DH. Antioxidative activity of gelatin hydrolysate increased in the gastrointestinal tract. The peptide with MW of 364 Da was the potential antioxidant peptide in the hydrolysate. Thus, ASF of hepatopancrease from Pacific white shrimp could be used as a replacer of commercial protease (Alcalase)

for production of hydrolysates from seabass skin with antioxidative activity. Also, skin hydrolysate can be used as a functional food ingredient.

5.7 References

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CHAPTER 6

Characteristics and antioxidative activity of carotenoprotein from shells of Pacific white shrimp extracted using hepatopancreas proteases

6.1 Abstract

Carotenoprotein from shells of Pacific white shrimp (*Litopenaeus vannamei*) were extracted with the aid of proteases from hepatopancreas of the same species at various levels (5-30 units/g shrimp shell) for different times (0-180 min). Recovery of carotenoprotein increased with increasing protease levels and hydrolysis times, but mainly reached the plateau at 120 min of hydrolysis. Carotenoprotein contained astaxanthin and astaxanthindiester as major carotenoids. Carotenoids extracted from carotenoprotein showed increasing ABTS, DPPH radical scavenging activities, FRAP and metal chelating activity when the concentrations increased up to 5 mg/mL (p<0.05). Carotenoprotein consisted of 73.58% protein, 21.87% lipids and 2.63% ash contents. It was rich in essential amino acids and Asp/Asn and Glu/Gln were found as dominant amino acids. Therefore, carotenoprotein from shell of Pacific white shrimp could be successfully extracted using shrimp hepatopancreas proteases and could serve as the value-added nutritive food ingredients or as the animal feed.

6.2 Introduction

Pacific white shrimp (*Litopenaeus vannamei*) accounts for 90% of the global aquaculture shrimp production. Thailand has been the world's leading exporter of cultivated shrimp since the mid-90s, (Lebel *et al.*, 2010). Pacific white shrimp and its products have become economically important for Thailand. By the year 2010, frozen Pacific white shrimp and products were manufactured and exported totally for 407,978 metric tons, particularly to USA and Japan (Lebel *et al.*, 2010). During shrimp processing, a large amount of by-products including cephalothorax, shell, etc. is generated. To exploit those left-over, cephalothoraxes have been used as raw material for production of shrimp hydrolysate, shrimp flavorant, carotenoid and chitin/chitosan (Armenta-López *et al.*, 2002; Bueno-Solano *et al.*, 2009; Gildberg and Stenberg, 2001; Kumar, 2000; Sowmya *et al.*, 2011). Shrimp shell has been

intensively used for carotenoprotein extraction (Armenta-López *et al.*, 2002; Babu *et al.*, 2008; Cano-Lopez *et al.*, 1987; Klomklao *et al.*, 2009; Sila *et al.*, 2012).

Carotenoid and carotenoproteins have been recognized as the natural colorant. Furthermore, they have been reported to have bioactivity, e.g. antioxidative, antimicrobial, etc (Sowmya and Sachindra, 2012). Carotenoids were extracted using traditional solvent extraction, supercritical fluid extraction (Babu *et al.*, 2008) and vegetable oils (Sowmya and Sachindra, 2012). To increase the extraction efficiency, hydrolysis process mediated by protease has been implemented. Cano-Cano-Lopez *et al.* (1987) reported the use of Atlantic cod trypsin or bovine trypsin for the extraction of carotenoproteins from shrimp processing wastes. Chakrabarti (2002) also used trypsin, pepsin and papain to extract carotenoprotein from brown shrimp waste.

Among all shrimp products, whole shrimp without hepatopancreas currently is of increasing demand. Along the process, hepatopancreas is removed by vacuum sucking machine. Shrimp hepatopancreas can be the major source of proteases, especially trypsin and chymotrypsin (Hernández-Cortés *et al.*, 1997; Sriket *et al.*, 2012). Protease in hepatopancreas from freshwater prawn actively hydrolysed various proteinaceous substrates (Sriket *et al.*, 2012). Appropriate hydrolysis condition could lead to the increasing extraction yield of carotenoprotein from shrimp shells. Therefore, the present study aimed to investigate the impact of hydrolysis using protease from hepatopancreas on the extraction of carotenoproteins from Pacific white shrimp and to characterize the resulting carotenoproteins.

6.3 Objective

To extract carotenoprotein from cephalothorax of Pacific white shrimp using proteases from Pacific white shrimp hepatopancreas.

6.4 Materials and methods

6.4.1 Chemicals

2,2-Azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt (ABTS), 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (Trolox) and 2,2-diphenyl-1-picryl hydrazyl (DPPH), 3-(2-pyridyl)-5-6-diphenyl-1,2,4-triazine-

4′,4″-disulfonic acid sodium salt (ferrozine) and N-α-Benzoyl-DL-arginine 4-nitroanilide Hydrochloride (BAPNA) were purchased from Sigma (St. Louis, MO, USA). Anhydrous sodium sulfate, ferric chloride and ferrous chloride were obtained from Merck (Darmstadt, Germany). Methanol, hexane, acetone, petroleum ether, hydrochloric acid and sulfuric acid were procured from Lab-Scan (Bangkok, Thailand). Astaxanthin was obtained from Dr. Ehrenstorfer GmbH (Augsburg, Germany).

6.4.2 Preparation of shells from Pacific white shrimp

Shells of Pacific white shrimp (*Litopenaeus vannamei*), obtained from the Sea wealth frozen food Co., Ltd., Songkhla province, Thailand, were transported to the Department of Food Technology, Prince of Songkla University, Hat Yai, Songkhla within 2 h. Upon arrival, the samples were washed with tap water, followed by drying in hot air oven (Binder FED115, Tuttlingen, Germany) for 6 h at 65°C. The dried samples were then ground to obtain the particle size of 1.0-2.0 mm. Ground shells were placed in polyethylene bag and kept in dark.

6.4.3 Preparation of crude extract from hepatopancreas of Pacific white shrimp

Pooled hepatopancreases of Pacific white shrimp were obtained from the Sea wealth frozen food Co., Ltd., Songkhla province. Samples were placed in polyethylene bag and imbedded in a polystyrene box containing ice with an ice/sample ratio of 2:1 (w/w) during transportation for approximately 2 h. Upon arrival, the samples were stored at -18°C until use, but not longer than 1 month.

Prior to extraction of proteases, hepatopancreases of Pacific white shrimp were powdered in liquid nitrogen. Samples were then defatted by stirring in three volumes of acetone at -20°C for 30 min according to the method of Kishimura and Hayashi (2002). The homogenate was filtered in vacuo on Whatman No. 1 filter paper (Whatman International Ltd., Maidstone, England). The residue obtained was then stirred in two volumes of acetone at -20°C for another 30 min, and then the residue was left at room temperature until dried and free of acetone odour.

To prepare the crude extract, acetone powder was suspended in 10 mM Tris–HCl, pH 8.0 containing 1 mM $CaCl_2$ at a ratio of 1:50 (w/v) and stirred continuously at 4°C for 3 h. The suspension was centrifuged for 10 min at 4°C at 10,000×g to remove the tissue debris using a refrigerated centrifuge (Beckman Coulter, Avanti J-E Centrifuge, Fullerton, CA, USA). The supernatant was lyophilized using a SCANVAC CoolSafe™ freeze-dryer (CoolSafe 55, ScanLaf A/S, Lynge, Denmark). Before use, the lyophilized sample (10 g) was dissolved with 50 mL of cold distilled water (4°C) and the solution is referred to as "crude extract".

6.4.4 Assay for trypsin activity

Trypsin activity of crude extract was measured using BAPNA as a substrate according to the method of Khantaphant and Benjakul (2008). A 200 µL of sample was mixed with 200 µL of distilled water and 1000 µL of reaction buffer (50 mM Tris–HCl buffer, pH 8.0, containing 10 mM $CaCl_2$). The reaction was initiated by adding 200 µL of 2 mg/mL BAPNA to the reaction mixture. After incubation for 20 min at 60°C, 200 µL of 30% acetic acid (v/v) were added to terminate the reaction. Production of *p*-nitroaniline was measured by monitoring the absorbance of reaction mixture at 410 nm (A_{410}) using a UV-1601 spectrophotometer (Shimadzu, Kyoto, Japan). A blank was conducted in the same manner except that the sample was added after addition of 30% acetic acid. One unit was defined as the amount of trypsin causing an increase of 1.0 in A_{410} per min.

6.4.5 Extraction of carotenoprotein using crude extract from hepatopancreas

Carotenoprotein from shrimp shell was extracted with the aid of crude extract from hepatopancreas. Ground shrimp shell (25 g) was mixed with five volumes of distilled water adjusted to pH 8 using 1.0 M NaOH and pre-incubated at 60°C. The mixture was added with crude extract from hepatopancreas to obtain different levels of protease (5, 10, 20 and 30 units/g shrimp shell). The mixtures were shaken continuously at 60°C for various times (0, 30, 60, 120, and 180 min). The control was performed in the same manner without crude extract. At the time

designated, the mixtures were heated at 95°C for 3 min to inactivate proteases. The samples were then filtered through two layers of cheesecloth. The filtrate containing carotenoprotein was adjusted to pH 4.5 with 2 M HCl. The precipitate was recovered by centrifugation at 5,000×g for 30 min at 4°C. The pellet obtained was dissolved in cold distilled water and dialyzed with 20 volumes of distilled water with 3 changes within 12 h. The samples were lyophilized and dry matter was referred to as "carotenoprotein.". All samples were determined for protein recovery and total carotenoid content.

6.4.5.1 Measurement of protein recovery

Carotenoprotein samples (2.5 g) were mixed with 10 mL of 0.5 M NaOH, followed by incubating at 85°C for 1 h. The mixture was centrifuged at 8,000 ×*g* for 10 min. The protein contents of supernatant were measured by the Biuret method (Robinson and Hogden, 1940) using bovine serum albumin as a standard. To obtain the total protein in shell, ground shells (2.5g) were dissolved in 10 mL of 0.5 M NaOH for 1 h at 85°C, followed by centrifugation at 8,000×*g* for 10 min (Klomklao *et al.*, 2009). Protein recovery was expressed as the percentage of proteins in the extracted carotenoprotein, relative to total proteins of shrimp shells.

6.4.5.2 Determination of total carotenoid content

Total carotenoid content was determined according to the method of Simpson and Haard (1985) with a slight modification. The carotenoprotein sample (1 g) was homogenized in 25 mL of cold acetone (-20°C) for 2 min using a Polytronhomogenizer (PT-MR 3,000, Littau, Switzerland) at a speed of 13000 rpm and the homogenate was filtered through a Whatman No. 1 filter paper under vacuum. The filtrate was placed in a separator funnel and was partitioned with 25 mL of petroleum ether. The separating funnel containing sample/solvent mixture was shaken gently and was allowed to stand at room temperature (25°C) for 10 min. The lower layer was drawn off. The top layer was washed twice with 25 mL of distilled water. The petroleum ether layer obtained was dried by occasional shaking with 15 g of anhydrous sodium sulfate for 30 min. The dried sample was filtered through a coarse

sintered glass funnel. The residual sodium sulfate was then washed with small volumes of petroleum ether for several times to remove all pigments. All petroleum ether fractions were pooled and evaporated under vacuum at 50°C using an EYELA rotary evaporator N-1000 (Tokyo Rikakikai, Co. Ltd, Tokyo, Japan). The residue was dissolved in petroleum ether and made up to a final volume of 10 mL. After being appropriately diluted, the absorbance was read at 468 nm. The content (C) of carotenoid in carotenoprotein sample was calculated using the equation given by Saito and Regier (1971):

$$C\ (\mu g/g\ sample) = \frac{A_{468} \times \text{volume of extract} \times \text{dilution factor}}{0.2 \times \text{weight of sample used in gram}}$$

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

6.4.6 Characterisation of carotenoprotein

Carotenoprotein extracted under an optimal condition, yielding the highest protein recovery and carotenoid content, was subjected to analyses, in comparison with shrimp shell.

6.4.6.1 Proximate analyses

Protein was determined by the Kjeldahl method using a conversion factor of 6.25 (AOAC, 2000). Ash and fat contents were determined using a furnace muffle at 600°C and with the Soxhlet apparatus,respectively, as per the method of AOAC (2000). The contents were expressed on a dry weight basis.

6.4.6.2 Determination of chitin content

Chitin content was determined according to the method of Spinelli *et al.* (1974) as modified by Simpson et al. (1985). Samples (2 g) were mixed with 30 mL of 2% (w/v) NaOH at 25°C for 6 h. The mixture was filtered under vacuum using a Whatman No. 1 filter paper. The residue was shaken with 15 mL of 1 M HCl for 30 min at 25°C, filtered and washed with deionized water. The washed residue was then homogenized with cold acetone (-20°C) at a speed of 13000 rpm a Polytron homogenizer for 3 min to remove the pigment. After washing with 3 volumes of

deionized water, 30 mL of 0.3% NaOCl was mixed with the sample and stirred for 6 h at 25°C. The mixture was then filtered and washed with deionized water. The residue was dried at 60°C for 24 h using an oven (Memmert, Schwabach, Germany) and the dried matter was referred to as "chitin".

6.4.6.3 Color measurement

Color was measured using a colorimeter (HunterLab, Model colorFlex, VA, USA). The color was reported in CIE system. L^*, a^* and b^* parameters indicate lightness, redness/greenness and yellowness/blueness, respectively.

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

Protein patterns of carotenoprotein samples extracted with or without proteases from hepatopancreas were determined using SDS-PAGE according to the method of Laemmli (1970).

6.4.6.5 Determination of amino acid composition

Amino acid composition of shrimp shell and carotenoprotein were analyzed according to the method of Ganno *et al.* (1985) with a slight modification. The samples were hydrolyzed under reduced pressure in 4 M methanesulphonic acid containing 0.2% (v/v) 3-2(2-aminoethyl) indole at 115°C for 24 h. For analyzing the tryptophan content, the samples were hydrolyzed by 3 N mercaptoethanesulphonic acid to avoid the decomposition of tryptophan (Penke *et al.*, 1974). The hydrolysates were neutralized with 3.5 M NaOH and diluted with 0.2 M citrate buffer (pH 2.2). An aliquot of 0.04 mL was applied to an amino acid analyzer (MLC-703; Atto Co., Tokyo, Japan).

6.4.6.6 Preparation and characterization of carotenoids extracted from carotenoprotein

Carotenoprotein (50 g) was extracted by homogenising the sample with 50 mL of acetone. The extract was filtered using a filter paper No.1. The residue

was repeatedly extracted with fresh solvent until it was colourless. The extracts were pooled together and were phase-separated with an equal quantity of hexane. The hexane extract was repeatedly washed with an equal quantity of 0.1% saline to remove traces of acetone if any. The extract was dried with 12.5 g of sodium sulphate, filtered and then evaporated under vacuum at 40°C using a rotary evaporator. The residual solvent was removed by flushing with nitrogen gas. The extracted carotenoids were subjected to determinations.

6.4.6.6.1 Thin-layer chromatography

The carotenoids were separated using thin-layer chromatography (TLC) with activated 20 × 20 cm silica gel plates (Silica gel G. Merck, type 60, Darmstadt, Germany) following the modified procedure described by Sánchez-Camargo et al. (2011). The extracted carotenoids were applied onto the plates and the separation was carried out using a mobile phase (acetone/hexane, (25:75%, v/v)). *β*-carotene and astaxanthin were used as standards. Rf of sample bands were calculated.

6.4.6.6.2 Determination of antioxidative activities

Extracted carotenoid solutions with different concentrations (1, 2, 3, 4 and 5 mg/mL) were prepared. Those solutions were subjected to determination of antioxidative activities.

- **DPPH radical scavenging activity**

DPPH radical scavenging activity was determined as described by Wu *et al.* (2003) with a slight modification. Sample (1.5 mL) was added with 1.5 mL of 0.15 mM DPPH in 95% methanol. 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 spectrophotometer. 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.

- ABTS radical scavenging activity

ABTS radical scavenging activity was determined as described by Binsan *et al.* (2008). The stock solutions included 7.4 mM ABTS solution and 2.6 mM potassium persulfate solution. The working solution was prepared by mixing two stock solutions in equal quantities and 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 A_{734} of 1.1 ± 0.02 using a spectrophotometer. Fresh ABTS solution was prepared daily. Sample (150 µL) 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 the spectrophotometer. A standard curve of Trolox ranging from 50 to 600 µM was prepared. The activity was expressed as µmol Trolox equivalents (TE)/mL.

- Ferric reducing antioxidant power (FRAP)

FRAP was assayed according to Benzie and Strain (1996). Stock solutions included 300 mM acetate buffer (pH 3.6), 10 mM TPTZ (2,4,6-tripyridyl-*s*-triazine) solution in 40 mM HCl, and 20 mM $FeCl_3 \cdot 6H_2O$ solution. A working solution was prepared freshly by mixing 25 mL of acetate buffer, 2.5 mL of TPTZ solution and 2.5 mL of $FeCl_3 \cdot 6H_2O$ solution. The mixed solution was incubated at 37°C for 30 min and was referred to as FRAP solution. A sample (150 µl) was mixed with 2850 µl of FRAP solution and kept for 30 min in dark. The ferrous tripyridyltriazine complex (coloured product) was measured by reading the absorbance at 593 nm. The standard curve was prepared using Trolox ranging from 50 to 600 µM. The activity was expressed as µmol Trolox equivalents (TE)/mL.

- **Chelating activity towards Fe^{2+}**

Chelating activity towards Fe^{2+} was measured as per the method of Thiansilakul *et al.* (2007). The sample was mixed with 0.1 mL of 2 M $FeCl_2$ and 0.2 mL of 5 M ferrozine. The reaction mixture was allowed to stand for 20 min at room temperature (26–28°C). The absorbance was then read at 562 nm using a spectrophotometer. The blank was prepared in the same manner except that distilled water was used instead of the sample. The Fe^{2+} chelating activity was expressed as EDTA equivalents (EE)/mL. A standard curve of 0–50 μM EDTA was used.

6.4.7 Statistical analysis

Experiments were run in triplicate using three different lots of samples. Data were subjected to analysis of variance (ANOVA). Comparison of means was carried out by Duncan's multiple range test. For pair comparison, T-test was used (Steel and Torrie, 1980). Statistical analysis was performed using the Statistical Package for Social Science (SPSS 11.0 for windows, SPSS Inc., Chicago, IL, USA).

6.5 Results and discussion

6.5.1 Effect of different levels of crude protease and hydrolysis times on the extraction of carotenoprotein from shrimp shells

The effect of crude protease from Pacific white shrimp hepatopancreas at different levels and various hydrolysis times on the recovery of carotenoprotein from Pacific white shrimp shells is shown in Figure 16A. At the same hydrolysis time, the recovery of carotenoproteins increased with increasing protease levels ($p<0.05$). Nevertheless, no differences in recovery were noticeable when protease levels of 20 and 30 (units/g sample) were used ($p>0.05$). The limited amount of protein substrate for hydrolysis reaction by crude protease most likely contributed to the plateau observed when crude protease above 20 units/g shrimp shells was used. The results indicated that the addition of crude protease from Pacific white shrimp hepatopancreas was effective in increasing the recovery of carotenoproteins from shrimp shell. For all protease levels used, the rapid hydrolysis of protein was observed

within the first 60 min. Thereafter, a slower rate of hydrolysis was found up to 120 min. No differences in protein recovery were observed after 120 min ($p > 0.05$). This was in accordance with the slower rate of hydrolysis after 60 min. This typical curve was also reported by Klomklao *et al.* (2009) when trypsin from bluefish was used for recovery of carotenoproteins from black tiger shrimp shells. Furthermore, the increase in trypsin concentration (0–1.2 units/g shrimp shells) resulted in an increase in recovered proteins (Klomklao *et al.*, 2009). Trypsin was used to extract carotenoprotein from brown shrimp shell waste and showed the maximum recovery (55%) of carotenoid pigment in 4 h at (28±2°C). Pepsin and papain yielded 50% recovery when the same hydrolysis time was used (Chakrabarti, 2002). Armenta and Guerrero-Legarreta (2009) reported that fermented carotenoproteins from Pacific white shrimp waste were hydrolysed with a combination of protease and lipase. Protein recovery was used as an indicator for the cleavage of peptide bond, and the release of the carotenoprotein (Sila *et al.*, 2012).

When total carotenoid contents of carotenoproteins extracted from Pacific white shrimp shells without and with the aid of crude protease at different levels were determined (Figure 16B), similar trends were found to those of carotenoprotein recovery (Figure 16A). No marked changes in carotenoid content were observed after 60 min of hydrolysis ($p>0.05$). At the same hydrolysis time, total carotenoid contents of shrimp shells hydrolyzed with crude protease was higher than those without crude protease ($p<0.05$). Coincidentally, no differences in total carotenoid content were noticeable between carotenoproteins from Pacific white shrimp shells extracted with crude protease at 20 and 30 unit/g shrimp shell at all hydrolysis times ($p>0.05$). For carotenoproteins extracted using high protease levels (20 and 30 unit/g sample), carotenoid content decreased in both samples when hydrolysis time was more than 120 min ($p<0.05$). The decrease in the total carotenoid content with increasing hydrolysis time was presumably because of the decrease in stability of carotenoids. As a consequence, free carotenoids associated with protein could be more released and were prone to oxidation. Carotenoproteins from shrimp heads were recovered by autolysis at 50°C and pH 8.0. Highest carotenoid content in carotenoprotein was reported when autolysis was conducted for 4 h (Sowmya *et al.*, 2011). For carotenoprotein extracted from brown shrimp shell, trypsin showed higher

recovery of carotenoid than pepsin and papain, when the same hydrolysis time was implemented (Chakrabarti, 2002). Cano-Lopez *et al.* (1987) reported that proteolytic enzymes were used to disrupt the protein–carotenoid bond, thus increasing carotenoid recovery. Therefore, the optimum conditions for carotenoprotein extraction by crude protease were 20 units/g shrimp shells with hydrolysis time of 120 min at 60°C.

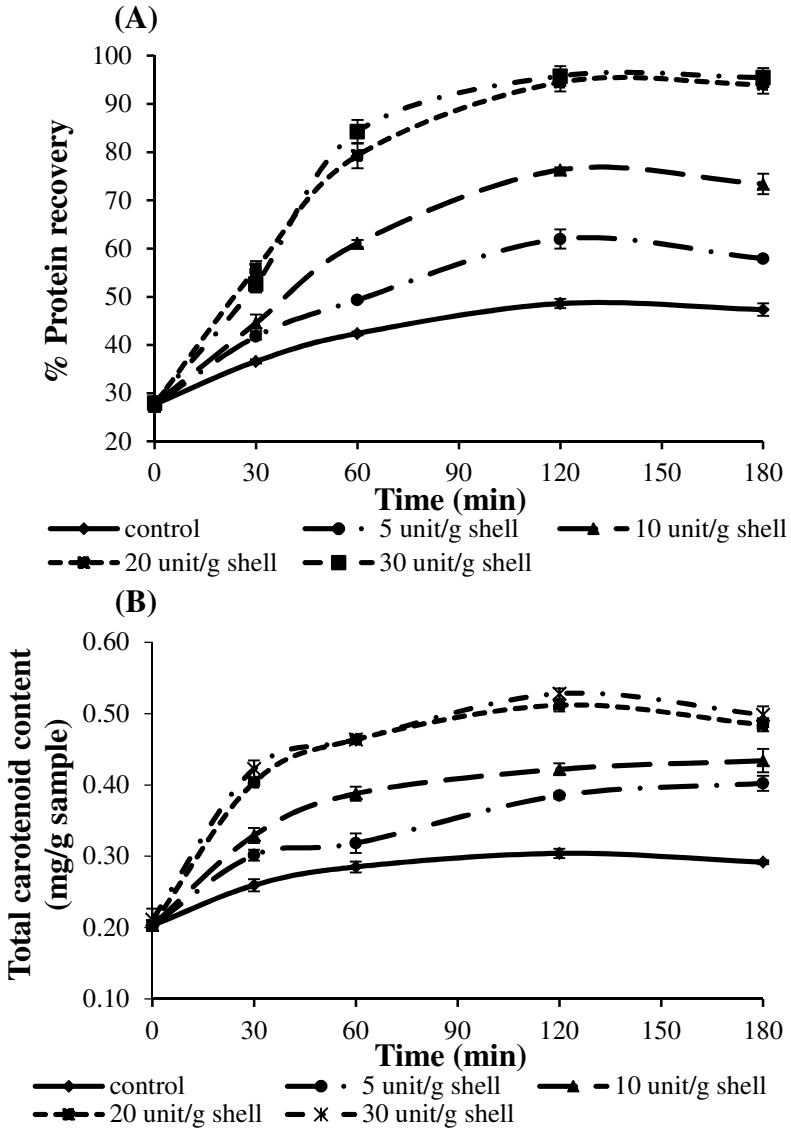

Figure16 % Protein recovery (A) and total carotenoid content (B) of carotenoproteins extracted from Pacific white shrimp shell without and with the aid of crude protease from hepatopancreas at different levels for various times. Bars represent SD (*n=3*).

6.5.2 Compositions of carotenoproteins extracted from Pacific white shrimp shells

6.5.2.1 Characteristics composition and color

Chemical compositions of the extracted carotenoproteins and shrimp shells are presented in Table 17. Carotenoproteins showed higher protein content (73.58%) than shrimp shell (43.89%). Furthermore, higher fat content was also observed in carotenoproteins (21.87%), compared with that found in shell. On the other hand, carotenoprotein had much lower ash and chitin contents than shrimp shell. Shell has been reported to contain protein, chitin and mineral, mainly $CaCO_3$ (Sini *et al.*, 2007). The protease-aided extraction process facilitated the release of protein, and lipid from shrimp shells, whilst minerals and chitin still retained in shell. Carotenoproteins recovered from black tiger shells with the aid of bluefish trypsin had 70.20% protein and 19.76% fat contents (Klomklao *et al.*, 2009). Chakrabarti (2002) also found that carotenoprotein from tropical brown shrimp shell waste extracted by trypsin had lower ash and chitin contents than shrimp waste. Thus, proteins in shell could be effectively extracted from shell with trypsin from hepatopancreas of Pacific white shrimp. Nevertheless, fat or lipoproteins could be co-extracted. Carotenoproteins are stable complexes, in which carotenoids are bound to a high density lipoprotein. Astaxanthin in crustaceans are mostly esterified to fatty acids (Armenta and Guerrero-Legarreta, 2009). Guillou *et al.* (1995) reported that astaxanthinin (*Pandalus borealis*) shrimp was presented as diester, monoester and free forms (76%, 20% and 4%, respectively, relative to total astaxanthin).

Freeze-dried carotenoprotein sample was light orange in colour with a^*-value of 34.65 and b^*-value of 41.75. Shrimp shell had the lower a^*- and b^*-values ($p<0.05$). Higher a^*- and b^*-values in carotenoprotein suggested that astaxanthin associated with protein was extracted to high extent from shell. Astaxanthin causes crustaceans to range in colour from green, yellow, blue to brown (Lorenz and Cysewski, 2000; Wade *et al.*, 2012). Astaxanthin, a red carotenoid, has been identified as the predominant pigment isolated from Brazilian redspotted shrimp waste (Sánchez-Camargo et al., 2011). The color and hue saturation of crustacean is

dependent on the amount of astaxanthin deposited (Ponce-Palafox et al.,2006). Due to the high content of carotenoid in carotenoprotein (Figure 16B), it was orange-red in colour and could be used as coloring agent in animal feeds. Canthaxanthin or astaxanthin should be regarded as a vitamin for salmon and all salmon diets should be added with canthaxanthin or astaxanthin at a level above 10 mg/kg dry feed to ensure the well-being of the animal (Christiansen *et al.*, 1995).

Table 17 Chemical compositions and color of Pacific white shrimp shell and carotenoprotein extracted with the aid of crude protease from hepatopancreas.

Compositions/colour	Shell	Carotenoprotein
Protein (% dry wt)	43.89±1.55	73.58±0.73
Fat (% dry wt)	1.53±0.08	21.87±0.12
Ash (% dry wt)	18.77±0.64	2.63±0.43
Chitin (% dry wt)	34.92±1.18	1.42±0.11
Color		
L^*	71.62±0.38	59.95±0.27
a^*	13.53±0.27	34.65±0.19
b^*	18.62±0.47	41.75±0.22

Means ± SD (n=3).

6.5.2.2 Protein Patterns

Protein patterns of carotenoprotein recovered with or without protease from hepatopancreas of Pacific white shrimp are shown in Figure 17. The apparent molecular weights (MW) of the major protein bands of carotenoprotein recovered without protease were estimated to be 93 and 45 kDa. Proteins with apparent MW of 77, 61, 57, 53, 34 and 32 kDa were also found in the sample. For carotenoprotein extracted with the aid of protease from hepatopancreas, band intensity of protein with MW of 93 kDa was decreased. It was noted that protein with MW of 45 kDa, which was more likely actin, totally disappeared when protease from hepatopancreas was used. The result suggested that actin was susceptible to hydrolysis by protease added. Furthermore, other proteins were also hydrolyzed to some degree as evidenced by the decrease in band intensity with coincidental increase in band intensity at dye front

(Simpson *et al.*, 1993) reported that carontenoproteins recovered from lobster (*Homarus americanus*) waste with the aid of bovine trypsin had the proteins with MW of 44 and 90 kDa as the major proteins. The MW of carotenoproteins of *Homarus gammarus* ranged from 48 to 90 kDa (Buchwald and Jencks, 1968).

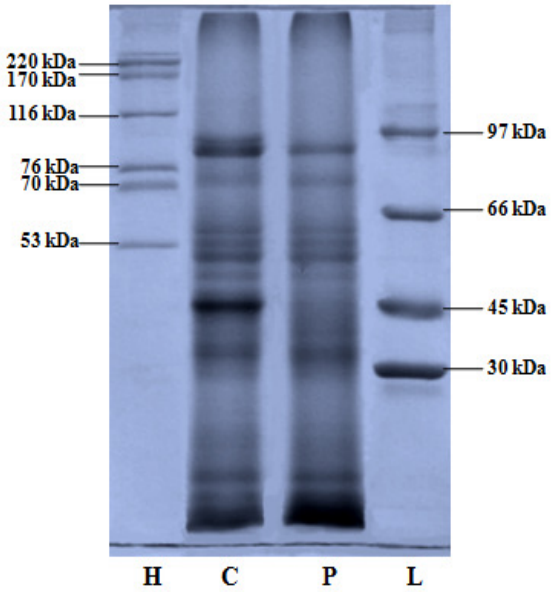

Figure 17 Protein patterns of carotenoprotein extracted from Pacific white shrimp shells H: high molecular weight standards; C: control (carotenoprotein extracted without protease); P: carotenoprotein extracted with protease; and L: low molecular weight standards.

6.5.2.3 Amino acid compositions

Amino acid compositions of Pacific white shrimp carotenoprotein and shells are presented in Table 18. Carotenoprotein extracted from Pacific white shrimp shells had higher total amino acid (604.97 mg/g) and total essential amino acid (234.46 mg/g) contents than those found in Pacific white shrimp shells (452.58 and 169.47 mg/g). Pacific white shrimp shells and carotenoprotein were rich in glutamic acid/glutamine (67.17 and 85.29 mg/g) and aspartic acid/asparagine (47.38 and 68.98 mg/g). Armenta and Guerrero-Legarreta (2009) reported that carotenoproteins extracted from fermented and non-fermented Pacific white shrimp waste were rich in aspartic acid and glutamic acids, leucine and lysine (128, 142, 111 and 100 mg/g, respectively). Carotenoprotein recovered with and without the aid of bluefish trypsin contained the high contents of glutamic acid/glutamine (13.00 and 13.25%) and

aspartic acid/asparagine (11.18 and 10.43%) (Klomklao *et al.*, 2009). Simpson and Haard (1985) also found that glutamic acid and aspartic acid were the dominant amino acids in carotenoproteinsisolated from shrimp wastes with and without the aid of bovine trypsin. However, carotenoprotein extracted from Pacific white shrimp shells had higher essential amino acid (leucine lysine, arginine, alanine, glycine, valine, phenylalanine, serine and theronine) contents than those found in Pacific white shrimp shells. Generally, low contents of cysteine and hydroxyproline were found in Pacific white shrimp shells and carotenoproteins.

Table 18 Amino acid compositions of shell and carotenoprotein from Pacific white shrimp

Amino acid compositions	**Shell (mg/g sample)**	**Carotenoprotein (mg/g sample)**
Asp+Asn	47.38	68.98
Hyp	0.99	1.39
Thr[A]	20.40	29.18
Ser	25.87	30.79
Glu+Gln	67.17	85.29
Pro	26.49	29.43
Gly	29.12	33.63
Ala	33.46	35.97
Cys	0.34	0.35
Val[A]	26.17	32.89
Met[A]	9.38	16.32
Ile[A]	18.98	30.35
Leu[A]	29.92	47.77
Tyr	19.82	29.27
Phe[A]	24.17	32.55
His[A]	11.17	15.15
Trp[A]	2.15	4.84
Lys[A]	27.14	40.33
Arg	31.94	39.98
Total amino acids	452.58	604.97
Total EAA[B]	169.47	234.46
Total NEAA[C]	283.11	370.51

[A] Essential amino acids in adults.
[B] Essential amino acids.
[C] Non-essential amino acids.

6.5.2.4 Carotenoids and their antioxidative activity

Carotenoids from carotenoprotein extracted from of Pacific white shrimp shells showed six distinct bands with Rfs of 0.33, 0.43, 0.49, 0.71, 0.75 and 0.79 after being separated using TLC (Figure 18). The band with Rf of 0.33 corresponded to free astaxanthin. The orange bands having Rf 0.49 and 0.75 corresponded to astaxanthin monoester and astaxanthindiester, respectively (Bulletin-003, 1998). Thus, astaxanthins were present in carotenoprotein as free, mono and diester. TLC is a technique widely used to separate and purify carotenoids due to its simplicity, flexibility and low cost (Liu *et al.*, 2012). Shrimp pigmentation is caused by keto-carotenoids (astaxanthin). These keto-carotenoids contain a conjugated double bonds system, responsible for their colour.Astaxanthin has been shown to exhibit antioxidative activity, which is related to the number of conjugated double bonds (Choubert and Baccaunaud, 2006).

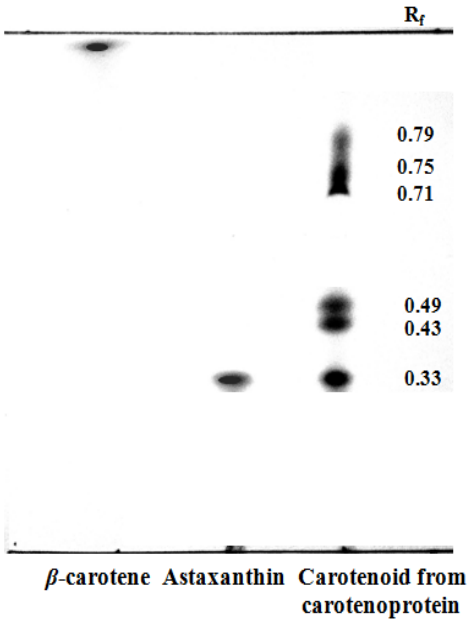

Figure 18 Thin layer chromatography of carotenoids extracted from carotenoproteins of Pacific whiteshrimp shells.

ABTS, DPPH radical scavenging activities, FRAP and metal chelating activity of carotenoids in carotenoprotein, extracted from Pacific white shrimp shell, increased as the concentrations increased up to 5 mg/mL ($p < 0.05$) (Figure 19). The results indicated that antioxidative activities of carotenoids in carotenoproteins of

Pacific white shrimp shell were in the concentration-dependent manner. Thus, carotenoids were capable of scavenging radicals and having the reducing power to a greater extent when higher concentrations were used. This result was in accordance with Sowmya and Sachindra (2012) who reported that carotenoid extracted from shrimp processing discards showed antioxidant activity. Crude extract rich in astaxanthin showed the strong antioxidant activity as indicated by radical scavenging, reducing activity and metal chelating activity, comparable to that of the known antioxidants α-tocopherol and TBHQ (Sowmya and Sachindra, 2012). Highest DPPH scavenging activity was reported for carotenoproteins from shrimp heads recovered by autolysis for 2 h at 50°C. The isolated carotenoprotein was found to have antioxidant activity with respect to singlet oxygen quenching, reducing power and metal chelating activity (Sowmya *et al.*, 2011). Furthermore, antioxidant activity of astaxanthin was reported to be 550 times higher than vitamin E and 6,000 times higher than vitamin C (Nishida *et al.*, 2007). Astaxanthinhas been known to quench, scavenge and trap free radicals in living organisms. Apart from electron donation, astaxanthin stabilizes these radicals by simply adding them to its own double-bonded chain (Higuera-Ciapara *et al.*, 2006). Each ionone of astaxanthin ring contains hydroxyl and keto moieties, which are capable of resonance-stabilising carbon-centered radical. Astaxanthin has been reported to prevent prooxidation of phospholipid membrane (Chávez-Crooker *et al.*, 2011). These terminal rings can scavenge radicals both at the surface and in the interior of the phospholipid membrane thereby, preventing membrane prooxidation (Goto *et al.*, 2001). Therefore, the carotenoids in carotenoprotein of Pacific white shrimp shells could exhibit antioxidative activity via radical scavenging or metal chelating mechanisms.

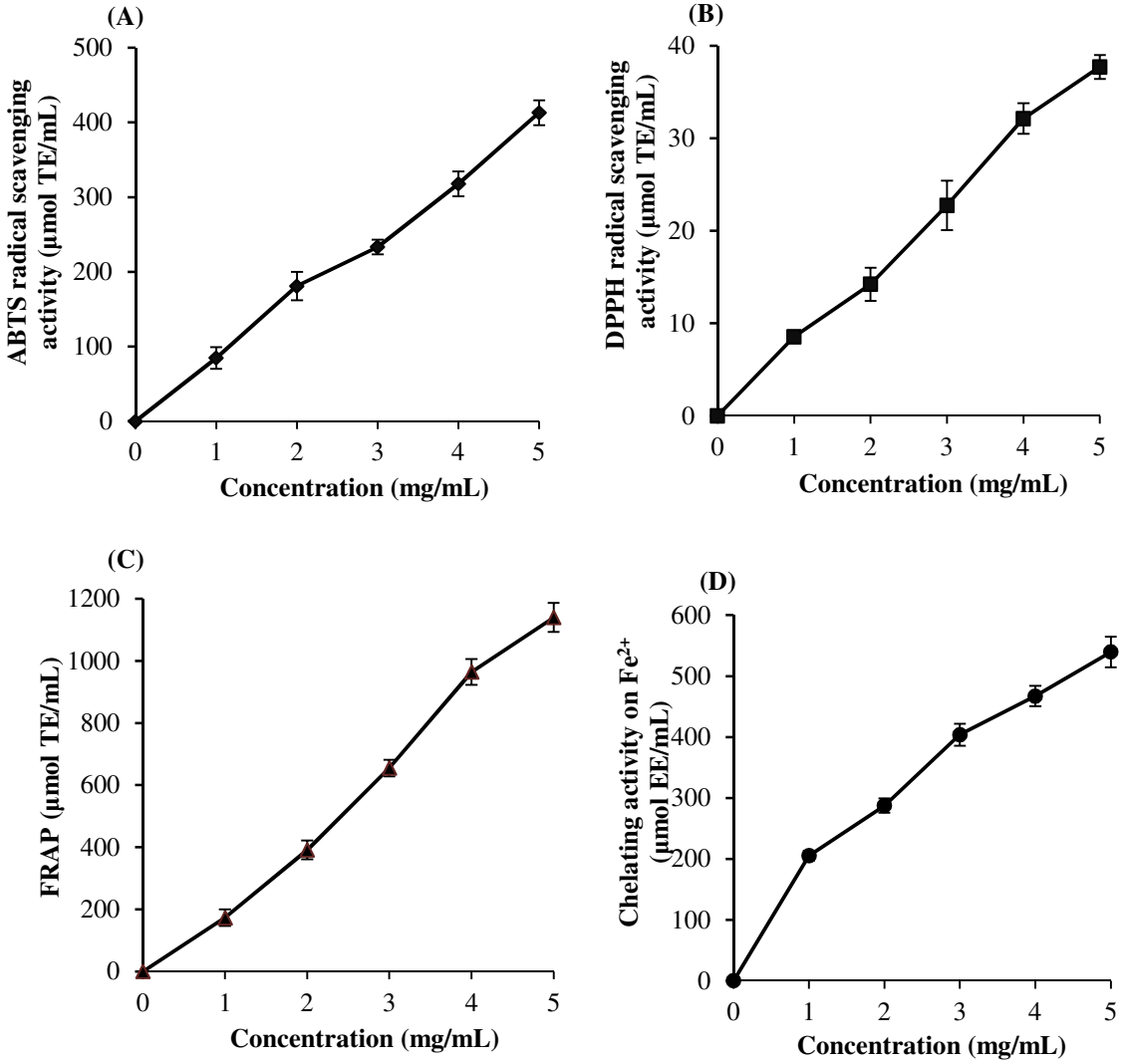

Figure 19 Antioxidative activities of carotenoids extracted from carotenoproteins of Pacific white shrimp shells at different concentrations as determined by ABTS radical scavenging activity (A) DPPH radical scavenging activity (B) FRAP (C) and metal chelating activity (D). Bars represent SD (*n=3*).

6.6 Conclusion

The optimum conditions for carotenoprotein extraction by crude protease from hepatopancreas of Pacific white shrimp were 20 units/g shrimp shells and hydrolysis time of 120 min at 60°C. Carotenoids extracted from carotenoprotein were rich in astaxanthin and showed strong antioxidant activity. Carotenoprotein had high contents of essential amino acids. Thus, protease from hepatopancreas of Pacific

white shrimp could be a potential novel enzyme for the recovery of carotenoprotein from shellfish wastes.

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CHAPTER 7

Compositions of lipids from striped catfish muscle extracted using protease from hepatopancreas of Pacific white shrimp

7.1 Abstract

Lipids from striped catfish muscle were extracted with the aid of crude protease extract (CPE) from hepatopancrease of Pacific white shrimp at different levels (5-15 unit/g protein) for various hydrolysis times (0-180 min) at 60°C. Yield of lipids increased within the first 30 min and was in agreement with increasing degree of hydrolysis. The increases in phospholipid and free fatty acid contents were observed with hydrolysis time up to 30 min (p<0.05). Yield of lipid increased when 50 mM $CaCl_2$ was combined with CPE. CPE exhibited similar extraction efficiency to Alcalase. However, the lower yield was obtained in comparison with the solvent extraction. Higher oxidation and hydrolysis were found in lipids extracted with the aid of CPE and Alcalase, compared with those found in lipid extracted using solvent (p<0.05). All lipid samples had triglyceride as the major component and were rich in palmitic acid and oleic acid.

7.2 Introduction

Thailand has become the leading country in the world market as a shrimp exporter. Total production of 550,147 metric tons with exportation value of 326,441 metric tons was reported in 2012. During shrimp processing, a large amount of by-products, including cephalothorax and shell, are generated (Takeungwongtrakul *et al.*, 2014). Shrimp hepatopancreas has been known as an excellent source of proteases, especially trypsin (Sriket *et al*., 2012). Currently, hepatopancreas removed from cephalothorax by a vacuum sucking machine was reported as an excellent source of proteases (Sriket *et al.*, 2011). Protease in hepatopancreas from freshwater prawns actively hydrolysed various proteinaceous substrates (Sriket *et al*., 2012)

Striped catfish (Pangasianodon hypophthalmus) or PlaSawai (in Thai) is one of the most important species in Thailand. Its meat has been popular amongst

the consumers worldwide (Singh *et al.*, 2011). Additionally, the meat contains a high amount of lipid (16.55±1.52%) (Begum *et al.*, 2012). Fish oil has been considered as an excellent source of long-chain *n*-3 polyunsaturated fatty acids (PUFAs), especially eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) (Linder *et al.*, 2005). PUFAs are of health benefit, e.g. reducing the serum cholesterol (Gbogouri *et al.*, 2006), etc. Fish oil can be produced by several methods including solvent extraction, supercritical fluid extraction and rendering (Gbogouri *et al.*, 2006). Those extraction methods have varying efficacy as determined by different extraction yield, operation cost, undesirable residues, etc. Enzymatic extraction using protease can serve as a promising alternative as the reaction can be carried out under mild conditions for short time (Mbatia *et al.*, 2010).

Muscle of some fish species, e.g. striped catfish, contains a high amount of lipids. Those lipids are generally removed prior to hydrolysis, in which the protein hydrolysate with high oxidative stability can be obtained (Klompong, *et al*, 2007). In general, solvents have been used for lipid removal and the residual solvent can be of safety concern for finished products. Recently, hepatopancreas was reported to contain the active proteases (Sriket *et al*., 2012). Those proteases can be used as the processing aid to increase the yield of oil extracted from striped catfish muscle. Simultaneously, protein hydrolysate can be attained as another value-added product. The present study aimed to investigate the impact of enzymatic extraction using crude protease extract from Pacific white shrimp hepatopancreas on the extraction efficiency and properties of lipids from the striped catfish muscle

7.3 Objective

To extract lipids from striped catfish muscle using protease from Pacific white shrimp hepatopancreas.

7.4 Materials and methods

7.4.1 Chemicals/enzyme

Palmitic acid, cupric acetate, *p*-anisidine, ammonium thiocyanate, cupric acetate and pyridine were purchased from Sigma (St. Louis. MO, USA).

Trichloroacetic acid, anhydrous sodium sulfate, isooctane and ferrous chloride were obtained from Merck (Darmstadt, Germany). 2-Thiobarbituric acid and 1,1,3,3-tetramethoxypropane were purchased from Fluka (Buchs, Switzerland). Methanol, ethanol, chloroform, petroleum ether, hydrochloric acid, sulfuric acid and ammonium thiocyanate were procured from Lab-Scan (Bangkok, Thailand). Alcalase (2.4L) was obtained from East Asiatic Co. (Thailand) Ltd. (Bangkok, Thailand).

7.4.2 Preparation of striped catfish

Fresh striped catfish (900–1000 g/fish) was purchased from a local market in Hat Yai, Songkhla province, Thailand. After transportation to the Department of Food Technology, Prince of Songkla University, Hat Yai, fish was immediately washed, filleted and mince to uniformity using a mincer with the hole diameter of 5 mm. The mince was stored on ice until use for lipid extraction.

7.4.3 Preparation of crude protease extract from hepatopancreas

Fresh hepatopancreas of Pacific white shrimp was collected from Sea wealth frozen food Co., Ltd., Songkhla province, Thailand. Hepatopancreas was packaged in polyethylene bag, stored in ice using a sample/ice ratio of 1:2 (w/w) and transported to the Department of Food Technology, Prince of Songkla University within 2 h. Upon arrival, The samples were cut into pieces with a thickness of 1–1.5 cm. Samples were ground into powder in the liquid nitrogen using a National Model MX-T2GN blender (Taipei, Taiwan) and homogenized in three volumes of acetone using a homogenizer (model PT-MR2100, POLYTRON®, KINEMATICA AG, Littau/Luzern, Switzerland) at 15,000 rpm at -20°C for 2 min according to the method of Kishimura and Hayashi (2002). The homogenate was stirred continuously using a magnetic stirrer model BIG SQUID (IKA®-Werke GmbH & CO.KG, Staufen, Germany) at -4°C for 30 min and filtered in vacuo on Whatman No. 4 filter paper (Whatman International Ltd., Maidstone, England). The residue obtained was then homogenised in two volumes of acetone (-20°C) for 2 min and stirred at 4°C for 30 min. The homogenate was filtered as described above. Then the residue was left at room temperature until dried and free of acetone odor.

To prepare the crude protease extract, acetone powder was suspended in an extraction buffer (10 mM Tris–HCl, pH 8.0 containing 1 mM $CaCl_2$) at a ratio of 1:50 (w/v) and stirred at 4°C for 3 h. The suspension was centrifuged for 10 min at 4°C at 10,000×g to remove the tissue debris using a refrigerated centrifuge (Beckman Coulter, Avanti J-E Centrifuge, Fullerton, CA, USA). The supernatant was lyophilized using a SCANVAC CoolSafe™ freeze-dryer (CoolSafe 55, ScanLaf A/S, Lynge, Denmark). The powder was referred to as "crude protease extract, CPE". Before use, the lyophilized sample was dissolved with cold distilled water (4°C) at a ratio of 1:5 (w/v). The solution was determined for protease activity and further used.

7.4.4 Protease activity assay

Protease activity of CPE and Alcalase were measured using casein as a substrate according to the method of An *et al.* (1994). The assay was performed at pH 8 and 60°C. One unit of activity was defined as that releasing 1 μmol of tyrosine per min (μmol Tyr/min).

7.4.5 Effect of protease levels and hydrolysis time on extraction of lipid

7.4.5.1 Enzymatic hydrolysis

Enzymatic extraction of lipid from striped catfish mince was preformed according to the method of Kechaou *et al.* (2009) with a slight modification. Striped catfish mince (50 g) was mixed with distilled water (25 mL) in a 250 mL- Erlenmeyer flask. The sample was homogenized at 15,000 rpm at 4°C for 1 min. The homogenate was adjusted to pH 8 using 2 M NaOH. Before hydrolysis, the mixture was pre-incubated in a water bath (Memmert, Bavaria, Germany) at 60°C for 10 min. The mixture was added with CPE to obtain different protease levels (5 10 and 15 units/g protein). Thereafter, the hydrolysis was allowed to proceed for different times (0, 30, 60, 120, 180 min). After hydrolysis, the protease was inactivated by heating at 90°C for 10 min in a water bath. The hydrolysates were determined for degree of hydrolysis and further used for lipid recovery.

7.4.5.2 Recovery of lipids

To recover the released lipids, the hydrolysates were transferred into the 200 mL tube and centrifuged at 9000×g at room temperature for 30 min. The tubes were put upright at -20°C for 2 h. Thereafter, the tubes were allowed to stand for 10 min at room temperature (28-30°C). Top phase rich in lipids turned to liquid, which was then collected using a pasteur pipette. The bottom phase, which was still solidified, was discarded. The collected lipids were then freeze-dried. Lipid samples were placed in a vial, flushed with nitrogen gas, sealed tightly and kept at -40°C until analysis. The yield was calculated and expressed as the percentage of lipids extracted, based on the weight of mince. Lipids were determinated for phospholipid and free fatty acid contents.

7.4.5.3 Determination of degree of hydrolysis

Degree of hydrolysis (DH) of hydrolysate was determined according to the method of Benjakul and Morrissey (1997). Hydrolysate samples with the 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 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 using a UV-1601 spectrophotometer (Shimadzu, Kyoto, Japan) and *α*-amino group was expressed in terms of *L*-leucine. The DH was defined as follows:

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

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

7.4.6 Effect of $CaCl_2$ on lipid extraction

To study the effect of $CaCl_2$, the homogenate of mince was prepared and adjusted to pH 8 as described previously. The $CaCl_2$ at different levels (0, 50, 100, and 200 mM) was added into the homogenate containing CPE at a level of 10

units/g protein. The mixtures were incubated at 60°C for 30 min, before termination by heat treatment (90°C for 10 min). Thereafter, the lipids were recovered as mentioned above. Yield of lipids was measured.

7.4.7 Comparative study of lipid extraction using different methods

Lipid extraction was carried out using CPE (10 units/g proteins+50 mM $CaCl_2$) or Alcalase (10 units/g proteins). The hydrolysis was performed at pH 8, 60°C for 30 min. The control was prepared in the same manner, except the enzyme was excluded. The lipids were collected as previously mentioned.

For solvent extraction, lipid was extracted by the Bligh and Dyer method (Bligh and Dyer, 1959). Sample (25 g) was homogenized with 200 mL of a chloroform: methanol: distilled water mixture (50:100:50, v/v/v) at the speed of 9500 rpm for 2 min at 4 ºC. The homogenate was added with 50 mL of chloroform and homogenized at 9500 rpm for 1 min. Thereafter, 25 mL of distilled water were added and homogenized at the same speed for 30 sec. The homogenate was centrifuged at 3000×*g* at 4 ºC for 15 min and transferred into a separating flask. The chloroform phase was drained off into the 125 mL Erlenmeyer flask containing 2-5 g of anhydrous sodium sulfate, shaken very well, and decanted into a round-bottom flask through a Whatman No.4 filter paper (Whatman International Ltd., Maidstone, England). The solvent was evaporated at 25 ºC using an EYELA rotary evaporator N-1000 (Tokyo Rikakikai, Co. Ltd, Tokyo, Japan) and the residual solvent was removed by nitrogen flushing.

The yield of all lipids extracted with different methods was calculated and expressed as the percentage of lipids. Lipid samples were collected in a vial, flushed with nitrogen gas, sealed tightly and kept at -40°C until analysis.

7.4.8 Analyzes

7.4.8.1 Measurement of peroxide value (PV)

PV was determined using the ferric thiocyanate method (Chaijan *et al.*, 2006). To 50 µL of lipid sample (10-fold dilution using 75% ethanol, v/v), 2.35 mL of 75% ethanol (v/v), 50 µL of 30% ammonium thiocyanate (w/v) and 50 µl of 20 mM

ferrous chloride solution in 3.5% HCl (w/v) were added and mixed thoroughly. After 3 min, the absorbance of the colored solution was read at 500 nm using a spectrophotometer. The blank was prepared in the same manner, except the distilled water was used instead of ferrous chloride. PV was calculated after blank subtraction. A standard curve was prepared using cumenehydroperoxide with the concentration range of 0.5–2 ppm. PV was expressed as mg cumenehydroperoxide/kg lipid.

7.4.8.2 Measurement of thiobarbituric acid-reactive substances (TBARS)

TBARS were determined as described by Buege and Aust (1978). Lipid sample (0.5 g) 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 ºC) for 10 min to develop a pink color, cooled with running tap water and centrifuged at 3600x*g* at 25 ºC for 20 min. 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 value was calculated and expressed as mg malonaldehyde/kg lipid.

7.4.8.3 Measurement of *p*-anisidine value (AnV)

AnV value of lipid sample was analyzed according to the method of AOCS (AOCS, 1990). Lipid sample (100 mg) was dissolved in 25 mL of isooctane. The solution (2.5 mL) was mixed with 0.5 mL of 0.5% (w/v) *p*-anisidine in acetic acid for 10 min. The absorbance was read at 350 nm using a spectrophotometer. The *p*-anisidine value was calculated using the following formula:

$$p\text{-anisidine value} = 25 \times \left(\frac{(1.2 \times A_2) - A_1}{W} \right)$$

where A_1 and A_2: the absorbance at 350 nm before and after adding *p*-anisidine, respectively; W= weight of sample (g)

7.4.8.4 Determination of conjugated diene (CD)

CD formed in the sample was measured as per to the method of Frankel, Huang, and Aeschbach (1997). Sample (0.1 mL) was dissolved in methanol (5.0 mL) and the absorbance at 234 was read. Content of conjugated diene was expressed as the increase in absorbance at 234 nm.

7.4.8.5 Measurement of free fatty acid

Free fatty acid (FFA) content was determined following the method of Lowry and Tinsley (1976). Lipid sample (0.1 g) was added with 5 mL of isooctane and swirled vigorously to dissolve the sample. The mixture was then treated with 1 mL of 5% (w/v) cupric acetate-pyridine reagent, prepared by dissolving 5 g of the reagent grade cupric acetate in 100 mL of water, filtering and adjusting the pH to 6.0-6.2 using pyridine. The mixture was shaken vigorously for 90 sec using a Vortex-Genie2 mixer (Bohemia, NY, USA) and allowed to stand for 20 sec. The upper layer was subjected to absorbance measurement at 715 nm. A standard curve was prepared using palmitic acid in isooctane at concentrations ranging from 0 to 50 μmol/5 mL. FFA content was expressed as g FFA/100 g lipid.

7.4.8.6 Determination of lipid compositions

Lipid compositions were determined using a thin layer chromatography/flame ionization detection analyzer (IATROSCAN® TLC/FID Analyser, IATRON Laboratories, Inc., Tokyo, Japan). The chromarods S-III (silica gel powder-coated Chromarod S-III, Iatron Laboratories Inc., Tokyo, Japan) were cleaned by soaking in 50% nitric acid solution in water and washed with tap water, distilled water and acetone. They were dried and scanned twice before use in order to remove possible contaminants from the rods. The lipid samples dissolved in chloroform was spotted on the rod. Lipid classes were separated using a double development procedure with the following solvent systems: n-hexane:diethylether:formic acid (50:20:0.3, v/v/v) for approximately 15 min, followed by hexane:benzene (1:1, v/v) for approximately 30 min. Then the rods were dried in an oven (105 ºC) for 10 min before being analyzed with the FID detector. The

analyses were carried out under the following conditions: flow rate of hydrogen, 150 mL/min; flow rate of air, 700 mL/min; scan speed, 30 s/scan. Peak area was quantitated and expressed as % of total lipid.

7.4.8.7 Determination of phospholipid content

Phospholipid content was measured based on the direct spectrophotometric measurement of complex formation between phospholipids and ammonium ferrothiocyanate as described by Stewart (1980). Lipids were dissolved in chloroform to obtain a final volume of 2 mL. One mL of thiocyanate reagent (a mixture of 0.10 M ferric chloride hexahydrate and 0.40 M ammoniumthiocyanate) was added. After thorough mixing for 1 min, the lower layer was removed and the absorbance at 488 nm was measured. A standard curve was prepared using phosphatidylcholine (0–50 ppm). The phospholipid content was expressed as mg/100 g lipid.

7.4.8.8 Determination of fatty acid profile

Fatty acid profile was determined as fatty acid methyl esters (FAMEs). FAMEs were prepared according to the method of AOAC (2000). The prepared FAMEs were injected to the gas chromatography (Shimadzu, Kyoto, Japan) equipped with the flame ionization detector (FID) at a split ratio of 1:20. A fused silica capillary column (30 m×0.25 mm), coated with bonded polyglycol liquid phase, was used. The analytical conditions were: injection port temperature of 250 °C and detector temperature of 270 ºC. The oven was programmed from 170 to 225 ºC at a rate of 1 ºC /min (no initial or final hold). Retention times of FAME standards were used to identify chromatographic peaks of the samples. Fatty acid content was calculated, based on the peak area ratio and expressed as g fatty acid/100 g lipid.

7.4.8.9 Fourier transform infrared (FTIR) analysis

FTIR analysis of lipids was performed in a horizontal ATR Trough plate crystal cell (45° ZnSe; 80 mm long, 10 mm wide and 4 mm thick) (PIKE Technology, Inc., Madison,WI, USA) equipped with a Bruker Model Vector 33 FTIR

spectrometer (Bruker Co., Ettlingen, Germany). Prior to analysis, the crystal cell was cleaned with acetone, wiped dry with soft tissue and the background scan was run. For spectra analysis, the lipid sample (200 µl) was applied directly onto the crystal cell and the cell was clamped into the mount of the FTIR spectrometer. Spectra in the range of 4000–500 cm^{-1} (mid-IR region) with the automatic signal gain were collected in 16 scans at a resolution of 4 cm^{-1} and were ratioed against a background spectrum recorded from the clean empty cell at 25°C. Analysis of the spectral data was performed using the OPUS 3.0 data collection software program (Bruker Co., Ettlingen, Germany).

7.4.9 Statistical analysis

Experiments were run in triplicate using three different lots of samples. Data were subjected to analysis of variance (ANOVA). Comparison of means was carried out by Duncan's multiple range test. For pair comparison, T-test was used (Steel and Torrie, 1980). Statistical analysis was performed using the Statistical Package for Social Science (SPSS 11.0 for windows, SPSS Inc., Chicago, IL, USA).

7.5. Results and discussion

7.5.1 Effect of CPE levels and hydrolysis time on yield and composition of lipids from striped catfish mince

Yield of lipid extracted from striped catfish mince using CPE with different levels (5, 10 and 15 unit/g protein) is shown in Figure 20A. At the same hydrolysis time, higher yield was obtained when CPE with higher protease activity increased up to 10 unit/g protein ($p<0.05$). Nevertheless, no differences in yield was found as CPE at 15 unit/g protein was incorporated ($p>0.05$). As the hydrolysis time increased, yield of lipids increased up to 60 min when CPE at 10 unit/g protein was used ($p<0.05$). It was noted that the yield increased as hydrolysis time increased up to 120 min when CPE at 5 unit/g protein was used. Nevertheless, the use of CPE at a level of 15 unit/g protein did not increase the yield of lipids ($p>0.05$) (data not shown). The results indicated that the addition of CPE from Pacific white shrimp was effective in increasing the recovery of lipid from striped catfish mince. When

hydrolysis of proteins proceeded in the presence of CPE, lipids associated with muscle proteins could be more hydrolyzed and released a free lipid from the homogenate and located at the top after centrifugation. The similar phenomenon was reported for hydrolysis of cuttlefish and sardine viscera by Protamex™, Alcalase and Flavourzyme (Kechaou *et al.*, 2009). The upper layer consisted mainly of neutral lipids, whilst the emulsified phase contained a large amount of polar lipids (Kechaou et al., 2009). Oil recovery from salmon by-products hydrolyzed with Neutrase® 0.8L and Protamex™ was 14.4 and 14.6%, respectively. Lipids extracted from salmon heads by commercial protease had the yield of 85% (Dumay *et al.*, 2006). When the hydrolysis of 180 min was implemented, the lower yield was obtained, regardless of protease levels used. With increasing hydrolysis time, the released lipids might undergo emulsification with peptide or phospholipids, e.g. phosphatidylcholine, etc at a higher extent. This might lead to the lower yield of top layer rich in neutral lipids. Furthermore, neutral oil was plausibly emulsified with polar lipids and peptides, thus forming the emulsified fraction (Gbogouri *et al.*, 2006).

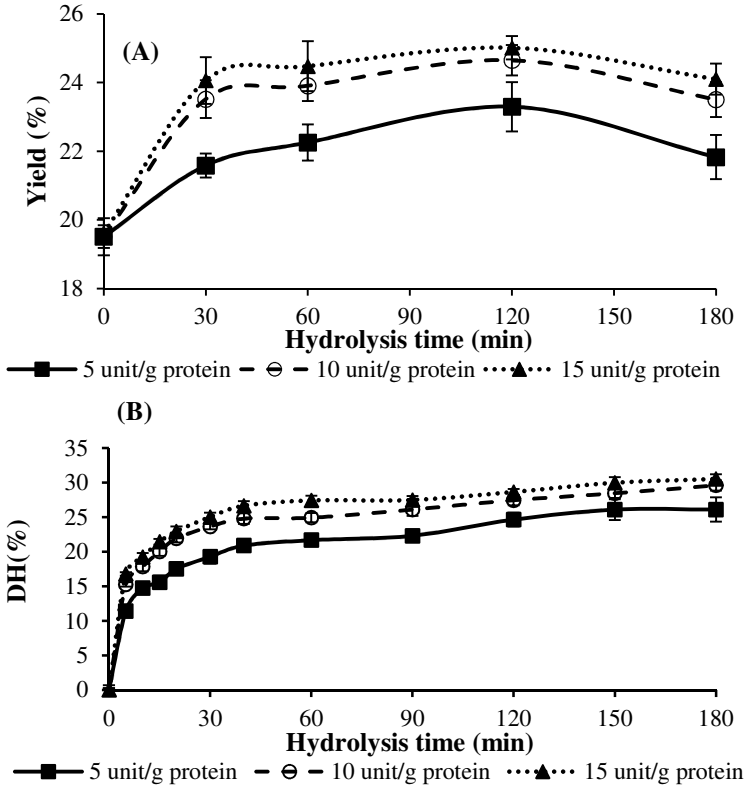

Figure 20 Yield (A) and degree of hydrolysis (B) of protein extracted from striped catfish mince with the aid of CPE at different levels for various times. Bars represent SD (*n=3*).

When considering the DH of protein in striped catfish mince, different DHs were noticeable as a function of time, depending on the protease levels (Figure 20B). Generally, hydrolysis rate was high within the first 60 min, followed by a slower hydrolysis rate. The rapid hydrolysis in the initial phase indicated that a large number of peptide bonds were hydrolyzed (Thiansilakul *et al.*, 2010). With increasing hydrolysis time, the hydrolysis rate was decreased, mainly due to a decrease in the available hydrolysis sites, enzyme autodigestion and/or product inhibition (Kristinsson and Rasco, 2000). It was noted that no marked differences in DH were found in samples containing CPE with 10 and 15 unit/g protein. This was in accordance with the similarity in extraction yield between systems containing CPE at 10 and 15 unit/g protein (Figure 20A). Thus, yield of oil extracted from striped catfish mince was related with the degree of hydrolysis. The result suggested that the cleavage of peptide bonds in mince allowed the lipids to be liberated to a higher extent.

Compositions of lipids extracted from striped catfish mince were monitored during hydrolysis. PL content of lipid extracted from striped catfish mince using CPE at 5 and 10 unit/g protein during hydrolysis of 180 min are shown in Figure 21A. Phospholipid content of lipids increased as the hydrolysis time increased up to 30 min ($p<0.05$). No changes in phospholipid content were found during 30 and 180 min of hydrolysis ($p>0.05$). Phospholipids are the essential lipid fraction in body tissue of fish (Lu *et al.*, 2011). Phospholipids, major components of cell membranes (Lu et al., 2011), are believed to readily prone to oxidative deterioration due to their highly unsaturated fatty acid composition (Thiansilakul *et al.*, 2010). Keriko *et al.* (2010) reported that phospholipid was the dominant component in lipids from *Cyprinus carpio*, *Cyprinus specularis*, *Micropterus salmoidesand Oreochromis leucosticus*. The increase in phospholipid content of lipids was in agreement of increased yield, especially during the first 30 min of hydrolysis time (Figure 21A). With sufficient hydrolysis, the phospholipids were more likely released. Higher content of phospholipid was found in lipids extracted with the aid of PCE at a level of 10 unit/g protein, compared with that using 5 unit/g protein ($p<0.05$).

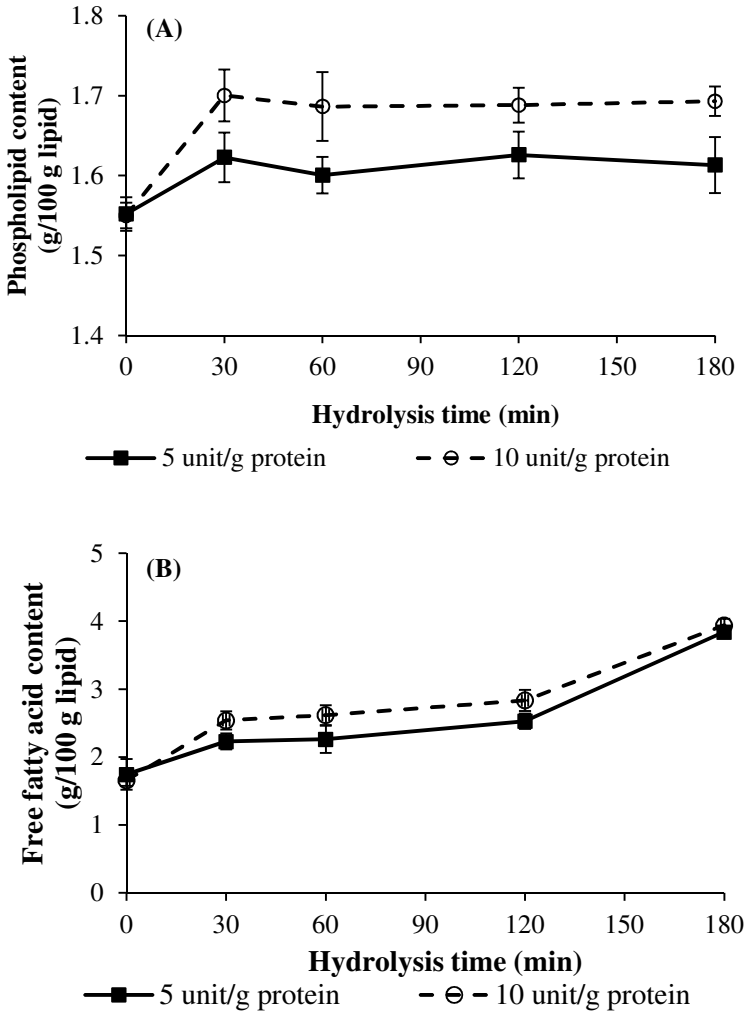

Figure 21 Changes in phospholipid (A) and FFA (B) content of lipid extracted from striped catfish mince with the aid of CPE at different levels for various times. Bars represent SD (*n=3*).

FFA contents of lipid extracted from striped catfish mince using CPE at 5 and 10 unit/g protein during hydrolysis are shown in Figure 21B. At the same hydrolysis time, FFA content was higher in lipids extracted with CPE having higher protease levels, except for 180 min, in which similar FFA content was found ($p>0.05$). FFA increased within the first 30 min of hydrolysis ($p<0.05$), followed by slight increase during 30-120 min ($p>0.05$). After 120 min of hydrolysis, the sharp increase in FFA content was obtained up to 180 min ($p<0.05$).The result suggested that the hydrolysis of ester bonds of triglyceride occurred to a higher degree with increasing hydrolysis time. Autoxidation of lipid is accelerated by the presence of free fatty acids, mono- and diacylglycerols (Choe and Min, 2006). The enhanced

hydrolysis of ester bonds plausibly proceeded at higher extent as induced by lipase or phospholipase localized in fish mince (Lu *et al.*, 2011). Lipids containing high level of free fatty acid are considered to be prone to oxidation, which were lowered with storage stability.

7.5.2 Effect of $CaCl_2$ on extraction yield

Yields of lipid extracted from striped catfish mince using CPE at 10 unit/g protein in the absence and presence of $CaCl_2$ at various levels (0, 50, 100, and 200 mM) were compared. Yield of lipid obtained using CPE without $CaCl_2$ was 21.0±0.7%. The increases in yield were noticeable as $CaCl_2$ of 50-200 mM was included, where the yields of 23.2-23.4% were achieved. It was noted that there were no differences in lipid recovery when $CaCl_2$ levels of 50, 100 and 200 mM were used (p>0.05). Calcium chloride was reported to activate trypsin in several fish (Klomklao, Benjakul, & Visessanguan, 2004). Trypsin was the dominant protease in hepatopancreas of Pacific white shrimp (Senphan et al., 2012). Proteinase activities of splenic extracts from three tuna species including skipjack tuna, yellowfin tuna and tongol tuna were increased with the addition of calcium chloride (Klomklao et al., 2004). Calcium protected trypsin against self-digestion and increased proteolytic activity (Klomklao *et al.*, 2004). Furthermore, Sipos and Merkel (1970) reported that calcium promotes the formation of a calcium–trypsin complex from a reversible inactive form (Klomklao *et al.*, 2004). The binding of calcium to trypsinogen induces a conformational change, which is associated with the formation of an active form. Therefore, calcium ions played an essential role in activation of protease from hepatopancrease of Pacific white shrimp. As a result, the activated protease in CPE with higher activity cleaved proteins more effectively. Simultaneously, lipids were more released as indicated by the increased yield.

7.5.3 Comparative study on different extraction methods

7.5.3.1 Yield

Yields of lipid extracted from striped catfish mince with CPE (10 unit/g protein) or Alcalase (10 unit/g protein) were compared with that of lipid extracted using solvent extraction. The yields were 23.5±0.5, 24.3±0.5 and 25.2±0.6%, respectively. Yield of lipid extracted without enzyme (control) was 19.5±0.5%. The use of CPE, Alcalase and solvent for lipid extraction from striped catfish mince resulted in a higher yield, compared with the control (without enzyme) (p<0.05). When hydrolysis proceeded by either protease in CPE or Alcalase, proteins associated with lipids, e.g. lipoproteins and protein-lipid complex were cleaved to a higher extent. As a result, lipids were liberated from proteins (Lipka *et al.*, 1995). Similar yields were observed between lipids extracted with the aid of CPE and Alcalase (p>0.05). Thus, CPE could be used to replace the commercial protease, in which the operation cost was reduced. It was noted that yield of lipids extracted by solvent was slightly higher than those extracted with the aid of protease, CPE or Alcalase. Bligh and Dyer method has become one of the most recommended methods for extracting lipids from tissues such as fish muscle. This method is known to extract more than 95% of total lipids (Iverson *et al.*, 2001). Linder *et al.* (2005) reported that yield of oil (17.4%) obtained from salmon head after 2 h of hydrolysis with Alcalase 2.4L was close to that obtained by the chemical extraction method. Salmon heads hydrolyzed with 0.5% (w/w) bromelain without water addition (1 h, 55°C) had an oil yield of 11.8 ± 0.4% g lipids/100 g wet weight (Mbatia *et al.*, 2010). For the control, the mince was subjected to incubation at 60°C for 30 min. The 'wet rendering' principle was introduced, in which heat could induce the disruption of fat cells as well as protein denaturation. As a result, the lipids could be released and the recovery of lipids could be achieved. Thus, the use of CPE could be a promising means to increase the extraction yield of lipids from striped catfish mince.

7.5.3.2 Lipid oxidation and hydrolysis

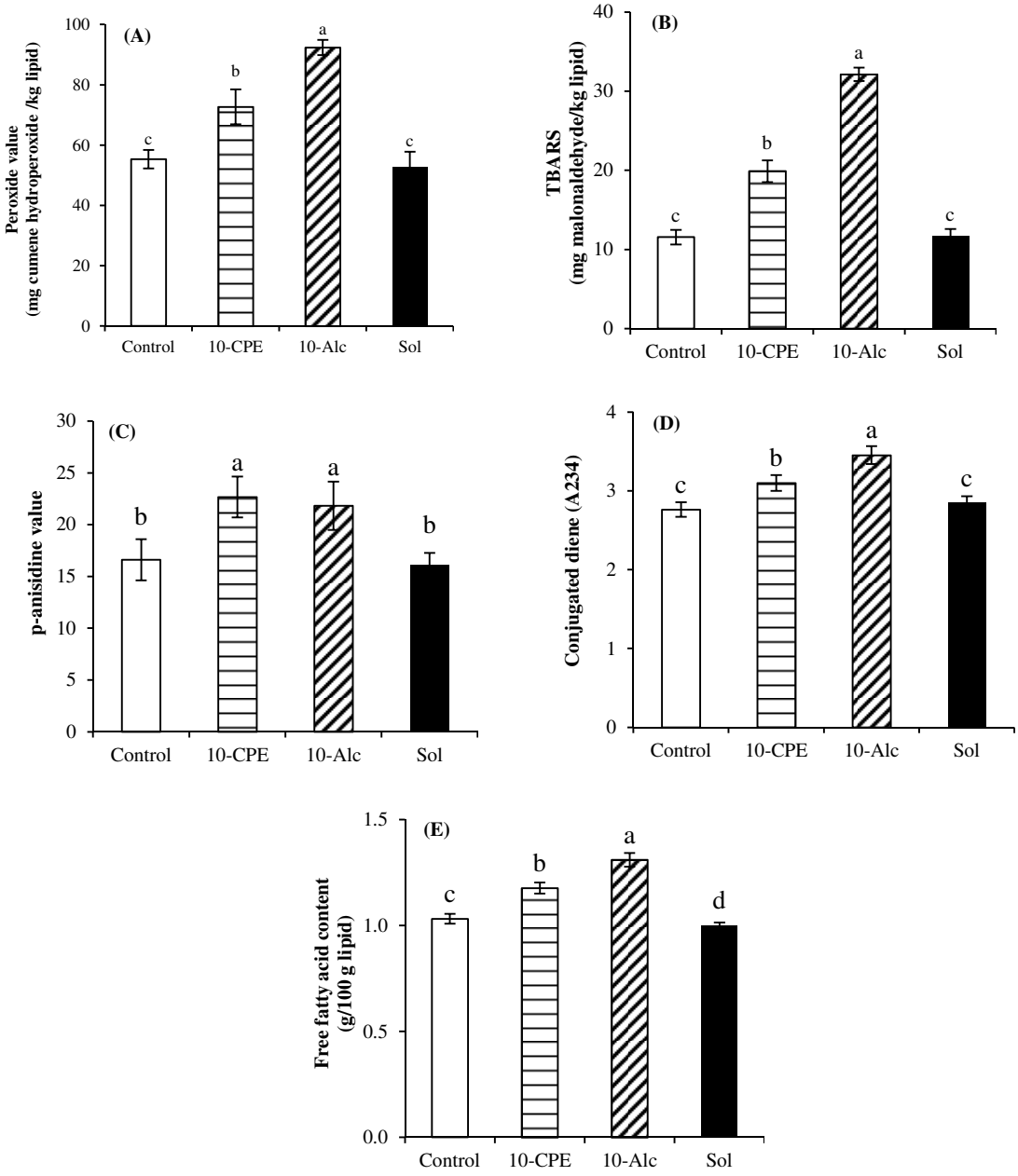

Figure 22 Peroxide values (A), TBARS values (B), *p*-anisidine values (C), conjugated diene (D) and free fatty acid content (E) of lipid extracted from striped catfish mince using different methods. Bars represent SD (*n=3*). Control: lipids extracted without enzyme at 60°C for 30 min; 10-CPE: lipids extracted with CPE (10 unit/g protein) at 60°C for 30 min; 10-Al: lipids extracted with Alcalase (10 unit/g protein) at 60°C for 30 min.; Sol: lipids extracted using the solvent (Bligh and Dyer method).

PV of lipid extracted from striped catfish mince using different extraction methods is presented in Figure 22A.The lipid extracted using Alcalase as the extraction aid showed the highest PV, followed by those extracted by CPE, the control and that with solvent extraction, respectively. However, there was no difference in PV of lipids extracted by solvent extraction and the control sample (p>0.05). The result suggested that lipid oxidation more likely took place in lipids during hydrolysis at 60 ºC for 30 min. At high temperature (60 ºC) used for hydrolysis, fatty acids likely underwent oxidation to a high extent. Autoxidation during the induction period can be retarded at low temperature (Choe and Min, 2006). The concentration of the hydroperoxides generally increases until the advanced stage of oxidation occurs. Formation of hydroperoxide is influenced by heat and some other factors such as light, oxygen, metal and fatty acid composition of oil (Chaijan *et al.*, 2006). Alcalase had higher efficiency in hydrolysis of muscle protein associated with lipid. As the result, lipids could be more released and those free lipids were more prone to oxidation as evidenced by higher PV. The result was in accordance with Hathwar *et al.* (2011) who reported that the higher PV in the oil recovered from fish visceral waste of catla (*Catlacatla*) and rohu (*Labeo rohita*) after hydrolysis at 40°C using Alcalase and Neutrase, in comparison with that found in oil from fresh fish viscera (Hathwar *et al.*, 2011).

TBARS of lipids as influenced by extraction methods (Figure 22B) showed similar trend with the PV results. The enhanced oxidation as indicated by higher TBARS was noticeable when striped catfish mince was subjected to hydrolysis at 60 ºC. TBARS value has been used to measure the concentration of relatively polar secondary reaction products, especially aldehydes (Chaijan *et al.*, 2006). The decomposition of hydroperoxides increases as the temperature increases (Tobolsky *et al.*, 1950). The hydroperoxides are decomposed to produce off-flavor compounds, leading to the poorer oil quality (Choe and Min, 2006). The result revealed that autoxidation of fatty acids in lipids took place during extraction with the aid of protease at high temperature.

AnV of lipid extracted from striped catfish mince varied, depending on extraction methods used. The lipid extracted using Alcalase and CPE showed higher AnV than the others (p<0.05). There was no difference in AnV of lipid extracted by

solvent method and the control (p>0.05). The *p*-anisidine reagent reacts with oxidation products, such as aldehydes (principally 2-alkenals and 2, 4-dienals), producing a yellowish product. An increased AnV indicates an increase in the amount of the non-volatile oxidation product (Choe and Min, 2006). Those aldehydes can be further oxidized or participate in dimerization or condensation reactions (Takeungwongtrakul *et al.*, 2014). It was suggested that the generation of non-volatile secondary product oxidation products occurred during hydrolysis, particularly when the hydrolysis was conducted at 60°C.

CD of lipid extracted by different methods (Figure 22D) had the same trend with PV and TBARS (Figure 22A and 22B). Almost immediately after hydroperoxides are formed, the non-conjugated double bonds that are present in natural unsaturated lipids are converted to conjugated double bonds (Chaijan et al., 2006). CD of lipids during microwave heating of fresh common carp and mackerel increased as the heating time increases for 0 to 24 min (Mahmoud *et al.*, 2009). CD formation in lipids therefore indicated the increased lipid oxidation when the CPE or Alcalase was used as the extraction aid.

FFA content of lipid extracted from striped catfish mince using different methods is presented in Figure 22E. The lipid extracted using Alcalase as the extraction aid showed higher FFA content than others (p<0.05).An increase in FFA is a well-established feature induced by enzymatic hydrolysis of esterified lipids (Lu *et al.*, 2011). At 60 C°, lipase or phospholipase were still active, leading to the enhanced lipid hydrolysis. Lipase or phospholipase from pollock muscle showed the optimal temperature of 37-42 C° (Audley *et al.*, 1978). Similarly, lipids extracted with CPE as the aid also showed high FFA content. Generally, free fatty acids formed were likely prone to oxidation. This was coincidental with the increased lipid oxidation as indicated by the increase in PV, TBARS, AnV and CD of lipid extracted using Alcalase and CPE, when compared with others. In the dark muscle, the majority of FFA was from TG but, in the lean tissue, FFA was mainly due to hydrolysis of phospholipids (Chaijan *et al.*, 2006). Kaneniwa *et al.* (2000) reported that enzymatic hydrolysis of lipids in fish muscle was reported in some lean and fatty fish such as cod, skipjack, carp, sardine, and rainbow trout. Thus, extraction methods affected the hydrolysis of lipids from striped catfish mince.

7.5.4 Lipid classes

Compositions of lipids from striped catfish mince extracted with various methods were different (Table19). Lipids in all samples were composed of triglyceride (TG) as the major constituent, followed by phospholipid (PL). TG constituted 97.17-93.48%, whilst PL ranged from 5.56 to 2.04%. The highest TG content was found in the control sample. Lipid from the control sample was also extracted at 60°C for 30 min. However, hydrolysis of proteins was much lower than that found in the systems containing CPE or Alcalase. The lower hydrolysis might not favour the release of the lipids. As a result, the lipids associated with proteins might not be exposed or available for hydrolysis by lipases or phospholipase. Higher PL content was observed in lipid extracted using solvent, compared with others (p<0.05). Higher amounts of free fatty acids (FFA) were noticeable in lipids extracted with the aid of CPE or Alcalase. During hydrolysis using CPE or Alcalase, TG decreased, whilst the FFA and MG increased (p<0.05). The result indicated that TG was more likely hydrolysed into free fatty acids, diglycerides and/or monoglycerides during hydrolysis for 30 min. Those decreases were in accordance with the increase in FFA content of lipid extracted using CPE and Alcalase (Figure 22C). Therefore, extraction methods had the influence on lipid composition.

Table 19 Composition of lipid extracted from striped catfish mince using different methods

Lipid sample	Compositions (% of total lipid content)				
	Triglyceride	**Free fatty acid**	**Diglyceride**	**Monoglyceride**	**Phospholipid**
Control	97.17±0.57a	0.52±0.02c	ND[a]	0.21±0.03c	2.04±0.22b
10-CPE*	96.26±0.24b	1.15±0.15b	0.16±0.01a	0.34±0.03b	2.07±0.12b
10-Alc	94.76±1.11c	1.43±0.07a	0.17±0.05a	0.54±0.05a	2.24±0.16b
Sol	93.48±0.62d	0.21±0.03d	0.17±0.02a	0.38±0.02b	5.56±0.30a

*Control: lipids extracted without enzyme at 60°C for 30 min; 10-CPE: lipids extracted with CPE (10 unit/g protein) at 60°C for 30 min; 10-Al: lipids extracted with Alcalase (10 unit/g protein) at 60°C for 30 min.; Sol: lipids extracted using the solvent (Bligh and Dyer method). Values are given as means ±SD (n=3).

[a] Different letters in the same column indicate significant differences (p< 0.05).

[b] ND, non-detecable.

7.5.5 Fatty acid profiles

Table 20 Fatty acid profile of lipid extracted from striped catfish mince using different methods

Fatty acids (g/100 g lipid)		Lipid samples			
		Control	**10-CPE**	**10-Alc**	**Sol**
Capric acid	C10:0	0.01±0.00	0.01±0.00	0.01±0.00	0.01±0.00
Lauric acid	C12:0	0.02±0.00	0.02±0.00	0.02±0.00	0.02±0.00
Tricosanoic acid	C13:0	0.01±0.00	0.01±0.00	0.01±0.00	0.01±0.00
Myristic acid	C14:0	3.54±0.04	3.54±0.02	3.50±0.00	3.47±0.00
Myristoleic acid	C14:1	0.03±0.00	0.03±0.00	0.03±0.00	0.03±0.00
Pentadecanoic acid	C15:0	0.33±0.00	0.33±0.00	0.33±0.00	0.32±0.00
Palmitic acid	C16:0	33.29±0.30	33.84±0.03	33.89±0.00	33.85±0.00
Palmitoleic acid	C16:1 n-7	2.72±0.05	2.72±0.03	2.72±0.00	2.63±0.00
Heptadecanoic acid	C17:0	0.72±0.00	0.74±0.00	0.74±0.00	0.72±0.00
cis-10-Heptadecenoic acid	C17:1	0.26±0.00	0.26±0.00	0.26±0.00	0.26±0.00
Stearic acid	C18:0	9.69±0.05	9.73±0.03	9.81±0.00	9.74±0.00
cis-9-Octadecenoic acid	C18:1 n-9	31.78±0.06	31.24±0.30	31.81±0.00	31.23±0.00
cis-9,12-Octadecadienoic acid	C18:2 n-6	3.46±0.02	3.46±0.01	3.42±0.00	3.40±0.00
cis-9,12,15-Octadecatrienoic acid	C18:3 n-3	3.29±0.05	3.29±0.02	3.24±0.00	3.20±0.00
cis-6,9,12-Octadecatrienoic acid	C18:3 n-6	0.19±0.00	0.20±0.00	0.20±0.00	0.19±0.00
Moroctic acid	C18:4 n-3	0.20±0.00	0.20±0.00	0.19±0.00	0.19±0.00
Arachidic acid	C20:0	0.11±0.00	0.11±0.00	0.11±0.00	0.11±0.00
cis-11-Eicosenoic acid	C20:1 n-9	0.80±0.00	0.89±0.01	0.89±0.00	0.06±0.00
cis-11,14-Eicosadienoic acid	C20:2 n-6	0.50±0.00	0.52±0.01	0.51±0.00	0.54±0.00
cis-8,11,14-Eicosatrienoic acid	C20:3 n-6	0.57±0.00	0.58±0.01	0.57±0.00	0.62±0.00
cis-11,14,17-Eicosatrienoic acid	C20:3 n-3	0.35±0.00	0.35±0.00	0.35±0.00	0.34±0.00
cis-5,8,11,14-Eicosatetraenoic acid	C20:4 n-6 (AA)	0.33±0.00	0.34±0.00	0.33±0.00	0.43±0.00
Eicosatetraenoic acid	C20:4 n-3	0.49±0.01	0.49±0.00	0.49±0.00	0.49±0.00
cis-5,8,11,14,17-Eicosapentaenoic acid	C20:5 n-3 (EPA)	0.30±0.00	0.30±0.00	0.29±0.00	0.33±0.00
Heneicosanoic acid	C21:0	0.04±0.00	0.03±0.00	0.03±0.00	0.040±0.00
cis-13-Docosenoic acid	C22:1 n-9	0.07±0.00	0.07±0.00	0.07±0.00	0.06±0.00
cis-7,10,13,16-Docosatetraenoic acid	C22:4 n-6	0.08±0.00	0.07±0.00	0.07±0.00	0.09±0.00
Docosapentaenoic acid	C22:5 n-6	0.12±0.00	0.12±0.00	0.12±0.00	0.16±0.00
cis-4,7,10,13,16,19-Docosahexaenoic acid	C22:6 n-3 (DHA)	0.46±0.01	0.46±0.00	0.46±0.00	0.60±0.00
Tricosanoic acid	C23:0	0.06±0.00	0.06±0.00	0.06±0.00	0.06±0.00
Lignoceric acid	C24:0	0.38±0.00	0.38±0.00	0.38±0.00	0.43±0.00
Unidentified peak	C10:0	3.70±0.00	3.82±0.01	3.85±0.00	3.98±0.00
Saturated fatty acids (SFA)		48.20±0.39	48.81±0.09	48.89±0.12	48.80±0.09
Monounsaturated fatty acids (MUFA)		35.65±0.16	35.20±0.33	35.78±0.36	34.27±0.06
Polyunsaturated fatty acids (PUFA)		10.34±0.11	10.37±0.06	10.23±0.04	10.58±0.04

[*] Control: lipids extracted without enzyme at 60°C for 30 min; 10-CPE: lipids extracted with CPE (10 unit/g protein) at 60°C for 30 min; 10-Al: lipids extracted with Alcalase (10 unit/g protein) at 60°C for 30 min.; Sol: lipids extracted using the solvent (Bligh and Dyer method). Values are given as means ±SD (n=3).

[a] Different letters in the same column indicate significant differences (p< 0.05).

[b] ND, non-detecable.

Fatty acid profiles of lipids extracted from striped catfish mince with different extraction methods are shown in Table 20. Lipids of all samples constituted PUFA of 10.23-10.58%, MUFA of 34.27-35.28% and SFA of 48.20-48.89%. There was no marked difference in fatty acid profiles between lipids extracted with different methods. All lipids samples contained palmitic acid as the most abundant SAT (33.25-33.89%). Oleic or cis-9-octadecenoic acid was the dominant MUFA (31.23-31.81%). In general, no differences in PUFAs were observed between all lipid samples. All lipids comprised linolenic acid or cis-9-12-15-octadecatrienoic acid ranging from 3.20 to 3.25%. Lipids extracted from striped catfish mince contained DHA and EPA of 0.29-0.33% and 0.46-0.60%, respectively. Chaijan, Jongjareonrak, Phatcharat, Benjakul, and Rawdkuen (2010) also found that palmitic acid was the most abundant fatty acid (about 30 g/100 g) in meat of raised giant catfish. Oleic acid (18:1) was the second major fatty acid in all types of giant catfish muscle. Generally, fatty acid composition of fish muscle is influenced by species of fish, the season, and wild or cultured types (Chaijan *et al.*, 2006).

7.5.6 FTIR spectra

FTIR spectra of lipid extracted from striped catfish mince using various methods are depicted in Figure 23. Dominant peaks were found at a wave number range of 3050–2800 cm^{-1}, representing CH stretching vibrations (cis C=CH, CH_2, CH_3 and CH_2/CH_3 stretching bands), which overlap with –OH group in carboxylic acids (3100–2400 cm^{-1}). Similar spectra between wavenumbers of 1500 and 1000 cm^{-1} were observed amongst all samples. Generally, the carbonyl (C=O) absorption of the triglyceride ester linkage was observed at 1742 cm^{-1}. Maximum frequency values ranging from 1741 to 1746 cm^{-1} were reported in lipids extracted from other fish species (Chaijan *et al.*, 2006; Thiansilakul *et al.*, 2010). Sánchez-Alonso *et al.* (2012) reported that the ester bond of hake lipids was found in the 1743–1740 cm^{-1}. It was noted that triglyceride containing ester bonds constituted more than 90% (Table 19). The peaks found at 1166 and 1237 cm^{-1} were associated with the stretching vibration of the C-O ester groups and the bending vibration of CH_2 groups, and the peaks observed at 1117 and 1095 cm^{-1} represented the stretching vibration of

the C-O ester groups (Takeungwongtrakul *et al.*, 2014). The (OH) band of hydroperoxides and/or oxidation- derivative hydroxyl compounds exhibit the characteristic absorption peak between 3600 and 3100 cm^{-1} (Sánchez-Alonso *et al.*, 2012). A peak representing hydroperoxide with wavenumber of 3466 cm^{-1} (–OH, –NH≡CH and =C–H) stretching was observed in all lipids. Nevertheless, the higher amplitude of band at 3466 cm^{-1} was observed with the sample using Alcalase as the extraction aid, indicating the presence of hydroperoxide in the sample. This was in agreement with higher PV for those samples (Figure 22A).

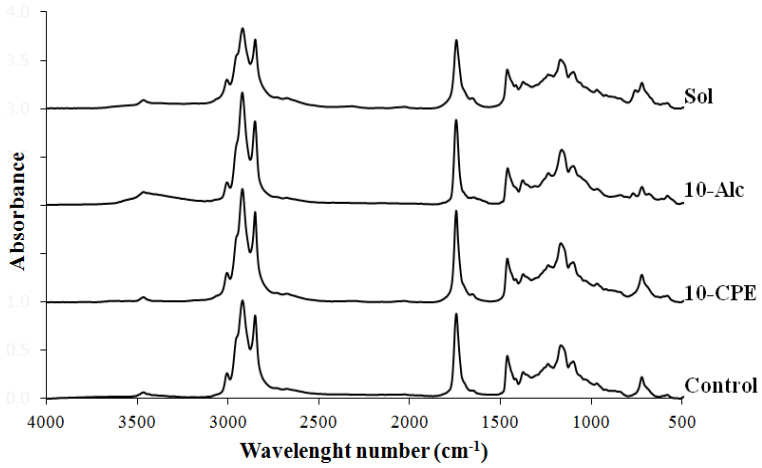

Figure 23 FTIR spectra of lipid extracted from striped catfish mince using different methods. Control: lipids extracted without enzyme at 60°C for 30 min; 10-CPE: lipids extracted with CPE (10 unit/g protein) at 60°C for 30 min; 10-Al: lipids extracted with Alcalase (10 unit/g protein) at 60°C for 30 min.; Sol: lipids extracted using the solvent (Bligh and Dyer method).

7.6. Conclusion

CPE at a level of 10 unit/g protein could be used as the extracting aid for lipid recovery from striped cat fish mince. However hydrolysis for 30 min at 60°C promoted the hydrolysis and oxidation of lipids to some extent. Lipids from striped cattfish mince can serve as the rich source of valuable nutrients for human consumption, espcially essential fatty acids.

7.7 References

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CHAPTER 8

Chemical compositions and properties of virgin coconut oil separated using protease from hepatopancreas of Pacific white shrimp

8.1 Abstract

Virgin coconut oil (VCO) was separated from coconut milk with the aid of crude protease extract (CPE) from hepatopancrease of Pacific white shrimp at different levels (5-15 unit/g protein) for various hydrolysis times (0-24 h). Yield of VCO increased within the first 6 h**.** The use of CPE exhibited similar separation efficiency to the typical fermentation process. However, lipids separated with fermentation process underwent oxidation to a higher extent, compared with those separated using CPE. CPE increased the creaming index and induced the collapse of oil droplets in coconut milk, as determined by the phase contrast microscope. VCO contained medium chain fatty acid (MCFA), especially lauric acid (C12:0), as the most abundant fatty acid, followed by myristic acid (C14:0). Therefore, VCO could be separated using CPE at room temperature for 6 h, in which the separation yield was increased and lipid oxidation was negligible.

8.2 Introduction

Coconut is another source of oil for food consumption and chemical applications. It is abundant in South East Asia, e.g. Thailand (Nakpong and Wootthikanokkhan, 2010). Virgin coconut oil (VCO) is prepared from fresh coconuts without chemicals or solvents at room temperature. Therefore, the oil retains its naturally occurring phyto-chemicals, which contribute to a distinctive coconut taste and smell. VCO is solidified at low temperature and becomes clear like water when liquefied (Marina *et al.*, 2009). The VCO contained high lauric acid, a medium chain fatty acid (MCFA). On the other hand, animal fats consist of long-chain saturated fatty acid (Handayani *et al.*, 2009). MCFAs are burned up immediately after consumption and therefore the body uses it immediately to make energy, instead of storing it as body fat (Enig, 1996).

VCO can be produced using several techniques as follows: (1) drying the freshly grated coconut meat at low temperature, no higher than 60°C, followed by pressing to extract the VCO; (2) by separating the coconut milk from the freshly grated coconut meat, followed by the addition of enzyme or aging for several hours, or by mechanical process using continuous centrifuge. The enzyme-assisted coconut oil separation significantly increased the oil yield in aqueous system (Tano-Debrah and Ohta, 1997). Enzymatic separation is effective to release the oil by breaking down the coconut emulsion. The efficiency of enzyme in separation of oil is influenced by substrate and enzyme concentration, temperature, pH, and incubation time for enzymatic reaction (Rahayu *et al.*, 2008). The basic principle of enzymatic of VCO production is to destabilize the emulsion, thus releasing oil (Rahayu *et al.*, 2008). Via enzymatic separation, simultaneous production of oil and hydrolyzed proteins can be achieved, in which both products can be further used. Furthermore, this process allows the removal of the impurities in the aqueous phase (Sant'Anna *et al.*, 2003).

Microbial proteases have been widely used for hydrolysis. However, cost of enzyme is another concern for user or industry. Thus, the proteases from cheap sources, especially fish processing byproducts, e.g. viscera can be alterative and promising for production of VCO. Shrimp hepatopancreas has been known to contain active proteases, especially trypsin (Sriket *et al.*, 2012). Currently, hepatopancreas removed from cephalothorax by vacuum sucking machine was reported as the excellent source of proteases (Senphan and Benjakul, 2012). The present study aimed to investigate the impact of crude protease extract from Pacific white shrimp hepatopancreas on separation efficiency and properties of VCO from coconut milk.

9.3 Objective

To separate virgin coconut oil from coconut oil from coconut milk using protease from Pacific white shrimp hepatopancreas.

8.4. Materials and methods

8.4.1 Chemicals

Palmitic acid, cupric acetate, *p*-anisidine, ammonium thiocyanate, cupric acetate and pyridine were purchased from Sigma (St. Louis. MO, USA). Trichloroacetic acid, anhydrous sodium sulfate, isooctane and ferrous chloride were obtained from Merck (Darmstadt, Germany). 2-Thiobarbituric acid and 1,1,3,3-tetramethoxypropane were purchased from Fluka (Buchs, Switzerland). Methanol, ethanol, chloroform, petroleum ether, hydrochloric acid, sulfuric acid and ammonium thiocyanate were procured from Lab-Scan (Bangkok, Thailand).

8.4.2 Preparation of coconut milk

Fresh milk from coconut of 'ordinary tall' cultivars (12–14 months' maturity) was purchased from a local market in Hat Yai, Songkhla province, Thailand. The mature coconuts were used for coconut milk preparation. Coconuts were subjected to deshelling, paring and removal of water. The white coconut kernel was grated using a rotary wedge cutter (Motor15, BSHatyai, Songkhla, Thailand). Coconut milk was prepared by pressing the grated coconut using a hydraulic press (Model stainless steel hydraulic press A2, Sakaya, Bangkok, Thailand). Coconut milk obtained was transported to the Department of Food Technology, Prince of Songkla University, Hat Yai, Songkhla and immediately used for VCO separation within 1 h. Coconut milk was analyzed for moisture, ash, fat and protein contents according to the method of AOAC (2000). The values were expressed as % (wet weight basis).

8.4.3 Preparation of crude protease extract from hepatopancreas

Fresh hepatopancreas of Pacific white shrimp was collected from Sea wealth frozen food Co., Ltd., Songkhla province, Thailand. Hepatopancreas was packaged in polyethylene bag, stored in ice using a sample/ice ratio of 1:2 (w/w) and transported to the Department of Food Technology, Prince of Songkla University within 2 h. Upon arrival, the samples were cut into pieces with a thickness of 1–1.5 cm. Samples were ground into powder in the liquid nitrogen using a National Model

MX-T2GN blender (Taipei, Taiwan) followed by defatting, in which the samples were homogenized in three volumes of acetone using a homogenizer (model PT-MR2100, POLYTRON®, KINEMATICA AG, Littau/Luzern, Switzerland) at 15,000 rpm at -20°C for 2 min according to the method of Kishimura and Hayashi (2002). The homogenate was stirred continuously using a magnetic stirrer model BIG SQUID (IKA®-Werke GmbH & CO.KG, Staufen, Germany) at 4°C for 30 min and filtered in vacuo on Whatman No. 4 filter paper (Whatman International Ltd., Maidstone, England). The residue obtained was then homogenized in two volumes of acetone (-20°C) for 2 min and stirred at 4°C for 30 min. The homogenate was filtered as described above. Then the residue was left at room temperature until dried and free of acetone odor.

To prepare the crude protease extract, the defatted sample was suspended in an extraction buffer (10 mM Tris–HCl, pH 8.0 containing 1 mM $CaCl_2$) at a ratio of 1:50 (w/v) and stirred at 4°C for 3 h. The suspension was centrifuged for 10 min at 4°C at 10,000×g to remove the tissue debris using a refrigerated centrifuge (Beckman Coulter, Avanti J-E Centrifuge, Fullerton, CA, USA). The supernatant was lyophilised using a SCANVAC CoolSafe™ freeze-dryer (CoolSafe 55, ScanLaf A/S, Lynge, Denmark). The powder was referred to as "crude protease extract, CPE". Before use, the lyophilized sample was dissolved with cold distilled water (4 °C) at a ratio of 1:5 (w/v). The solution was determined for protease activity and further used.

8.4.4 Protease activity assay

Protease activity of CPE was measured using casein as a substrate according to the method of An *et al.* (1994). The assay was performed at pH 8 and 60 °C. One unit of activity was defined as that releasing 1 μmol of tyrosine equivalent per min (μmol Tyr equivalent /min).

8.4.5 Separation of virgin coconut oil

8.4.5.1 Effect of enzymatic separation using CPE at different levels

Enzymatic separation of VCO from coconut milk was preformed according to the method of Raghavendra and Raghavarao (2010) with a slight

modification. The coconut milk (100 g) in a 250 mL-Erlenmeyer flask was adjusted to pH 8 using 2 M NaOH. Before hydrolysis, the mixture was added with CPE to obtain different protease levels (5 10 and 15 units/g protein). Thereafter, the hydrolysis was allowed to proceed at room temperature (28-30°C) for different times (0, 2, 4, 6, 8, 12, 16, 20 and 24 h). At the time designated, the mixtures were transferred into the 200 mL tubes and centrifuged at 4,900×g at room temperature for 30 min using Allegra^{TM} 25R centrifuge (Beckman Coulter, Palo Alto, CA, USA). Upper layer rich in oil was then collected using a pasteur pipette. The bottom (residue) and the middle (emulsion) phases were discarded. The collected oil was placed in a vial, flushed with nitrogen gas, sealed tightly and kept at -40°C until analysis. The yield of VCO was calculated and expressed as the percentage of oil separated, relative to total oil content as determined by the Gerber method (AOAC, 2000a).

8.4.5.2 Comparative study of VCO separation using different methods

VCO separation was carried out using CPE (10 units/g proteins). The hydrolysis was performed at pH 8 and 28-30 °C for 6 h. The control was prepared in the same manner, except that CPE was excluded. To prepare VCO by fermentation process, coconut milk was added with distilled water at 1:1 ratio (v/v). To 1 L of the mixture, 2.0 g of Baker's yeast (*Saccharomyces cerevisiae*) was added as an inoculum for the fermentation process (Mansor *et al.*, 2012). Mixture was stirred rigorously. The mixture was then left to stand for 36 h at room temperature. The upper oil layer was simply decanted using a pasteur pipette. All oils prepared by different methods were collected and stored at -40°C as previously described. All samples were calculated for yield and subjected to analyze.

8.4.6 Analyses

8.4.6.1 Color determination

Color of VCO was measured using a colorimeter (HunterLab, Model color Flex, VA, USA). The color was reported in CIE system. L^*, a^* and b^*-values indicate lightness, redness/greenness and yellowness/blueness, respectively.

8.4.6.2 Measurement of peroxide value (PV)

PV was determined using the ferric thiocyanate method (Low, 1978). To 50 µL of VCO sample (10-fold dilution using 75% ethanol, v/v), 2.35 mL of 75% ethanol (v/v), 50 µL of 30% ammonium thiocyanate (w/v) and 50 µl of 20 mM ferrous chloride solution in 3.5% HCl (w/v) were added and mixed thoroughly. After 3 min, the absorbance of the colored solution was read at 500 nm using a UV-1601 spectrophotometer (Shimadzu, Kyoto, Japan). The blank was prepared in the same manner, except the distilled water was used instead of ferrous chloride. PV was calculated after blank subtraction. A standard curve was prepared using cumenehydroperoxide with the concentration range of 0.5–2 ppm. PV was expressed as mg cumenehydroperoxide/kg oil.

8.4.6.3 Measurement of thiobarbituric acid-reactive substances (TBARS)

TBARS were determined as described by Buege and Aust (1978). VCO sample (0.5 g) 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 ºC) for 10 min to develop a pink color, cooled with running tap water and centrifuged at 3600x*g* at 25 ºC for 20 min. 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 value was calculated and expressed as mg malonaldehyde/kg oil.

8.4.6.4 Measurement of *p*-anisidine value (AnV)

AnV value of VCO sample was analyzed according to the method of AOCS (AOCS, 1990).

8.4.6.5 Determination of conjugated diene (CD)

CD formed in the sample was measured as per to the method of Frankel *et al.* (1997). Sample (0.1 mL) was dissolved in methanol (5.0 mL) and the

absorbance at 234 was read. Content of conjugated diene was expressed as the increase in absorbance at 234 nm.

8.4.6.6 Measurement of free fatty acid

Free fatty acid (FFA) content was determined following the method of Lowry and Tinsley (1976). VCO sample (0.1 g) was added with 5 mL of isooctane and swirled vigorously to dissolve the sample. The mixture was then treated with 1 mL of 5% (w/v) cupric acetate-pyridine reagent, prepared by dissolving 5 g of the reagent grade cupric acetate in 100 mL of water, filtering and adjusting the pH to 6.0-6.2 using pyridine. The mixture was shaken vigorously for 90 sec using a Vortex-Genie2 mixer (Bohemia, NY, USA) and allowed to stand for 20 sec. The upper layer was subjected to absorbance measurement at 715 nm. A standard curve was prepared using palmitic acid in isooctane at concentrations ranging from 0 to 50 μmol/5 mL. FFA content was expressed as g FFA/100 g oil.

8.4.6.7 Moisture content

VCO sample was analyzed for moisture content according to the method of AOAC (2000b)

8.4.6.8 Determination of fatty acid profile

Fatty acid profile of VCO samples was determined as fatty acid methyl esters (FAMEs). FAMEs were prepared according to the method of AOAC (2000). The prepared FAMEs were injected to the gas chromatography (Shimadzu, Kyoto, Japan) equipped with the flame ionization detector (FID) at a split ratio of 1:20. A fused silica capillary column (30 m×0.25 mm), coated with bonded polyglycol liquid phase, was used. The analytical conditions were: injection port temperature of 250 °C and detector temperature of 270 ºC. The oven was programmed from 170 to 225 ºC at a rate of 1 ºC /min (no initial or final hold). Retention times of FAME standards were used to identify chromatographic peaks of the samples. Fatty acid content was calculated, based on the peak area ratio and expressed as g fatty acid/100 g oil.

8.4.7 Effect of different separation processes on emulsion destabilization of coconut milk

Coconut milk with various separation processes or treatments was determined for emulsion stability and microstructure of oil droplets.

8.4.7.1 Measurements of emulsion stability

Creaming index, an indicator of emulsion stability, was measured according to the method of Raghavendra and Raghavarao (2010) with a slight modification. The samples were allowed to stand for 6 h at room temperature (28-30°C) to allow the separation into the cream (top) and the aqueous (bottom) phases. Total height of the emulsion in the test tube (HE) and the height of the aqueous layer (HS) were measured. The extent of creaming was expressed as creaming index, which was calculated using the following formula:

$$\text{Creaming index (\%)} = \frac{\text{HS}}{\text{HE}} \times 100$$

8.4.7.2 Microstructure of oil droplets

Oil droplets in coconut milk after different treatments were observed under phase contrast microscope (Model IX50; Olympus, Tokyo, Japan) equipped with camera. Samples were placed on a glass slide, covered with cover slip and observed at 400× magnification.

8.4.8 Statistical analysis

Experiments were run in triplicate using three different lots of samples. Data were subjected to analysis of variance (ANOVA). Comparison of means was carried out by Duncan's multiple range test. For pair comparison, T-test was used (Steel and Torrie, 1980). Statistical analysis was performed using the Statistical Package for Social Science (SPSS 11.0 for windows, SPSS Inc., Chicago, IL, USA).

8.5 Results and discussion

8.5.1 Proximate composition of coconut milk

Coconut milk had high moisture content (59.8±0.2%). Fat and protein contents were 33.4±0.5 and 5.4±0.4%, respectively. Low ash content (0.6±0.2%) was found. The result indicated that coconut milk could serve as a good source of oil. Coconut milk contained about 54% moisture, 35% fat and 11% solid non-fat (data not shown) (Simuang *et al.*, 2004; Tansakul and Chaisawang, 2006). The difference in fat contents might be associated with the process used, the maturation and variety of coconut, etc. The proteins in coconut milk have been known to function as emulsifier, which stabilizes the oil droplets in coconut milk.

8.5.2 Effect of CPE levels and hydrolysis time on yield of VCO from coconut milk

Yield of VCO separated from coconut milk using CPE with different levels (5, 10 and 15 unit/g protein) is shown in Figure 24. Within the first 2 h of hydrolysis, the increase in yield was observed for the coconut milk treated with CPE, regardless of CPE levels. However, no oil was separated from the coconut milk without CPE addition. No differences in yield were found amongst samples treated with various CPE levels ($p>0.05$). As the hydrolysis time increased, yield of VCO increased up to 6 h when CPE at 10 or 15 unit/g protein was used ($p<0.05$). In the presence of CPE at 5 unit/g protein, the yield increased gradually as hydrolysis time increased and reached the plateau at 16 h. It was noted that the use of CPE at a level of 15 unit/g protein did not increase the yield of VCO, in comparison with 10 unit/g protein ($p>0.05$) throughout the hydrolysis time of 24 h. Therefore, the addition of CPE from Pacific white shrimp at a sufficient amount was effective in increasing the recovery of oil from coconut milk. For the control (without CPE addition), the oil could be liberated gradually after 2 h and the highest yield (74.84%) was obtained when the coconut milk was allowed to stand at 28-30 °C for 16 h. Thereafter, no change in yield was noticeable up to 24 h ($p>0.05$). During incubation, the fermentation associated with the microbial growth might enhance the destabilization

of emulsion, plausibly mediated by microbial proteases. However, the proteolytic activity was most likely lower than that of CPE. Additionally, CPE was reported to exhibit the excellent hydrolytic activity towards protease (Senphan and Benjakul, 2012). Proteases in CPE were able to hydrolyze the proteins in coconut milk, which acted as emulsifying agent. When those proteins were cleaved, the emulsion was destabilized. This contributed to the higher release of free oil from coconut milk emulsion. The oil could be separated and located at the top after centrifugation. The similar phenomenon was reported for coconut milk emulsion subjected to hydrolysis by aspartic protease (Raghavendra and Raghavarao, 2010). Oil recovered from coconut milk hydrolyzed using papain had the yield of 60.09% (Mansor *et al.*, 2012). It was noted that CPE at higher level showed the higher efficacy in releasing oil from coconut milk. The concentration of enzyme greatly affects the hydrolysis rate, and thus influences the oil separation yield for a given period of time (Sant'Anna *et al.*, 2003). Thus, VCO could be separated from coconut milk with the aid of CPE at an appropriate level (10 unit/g protein).

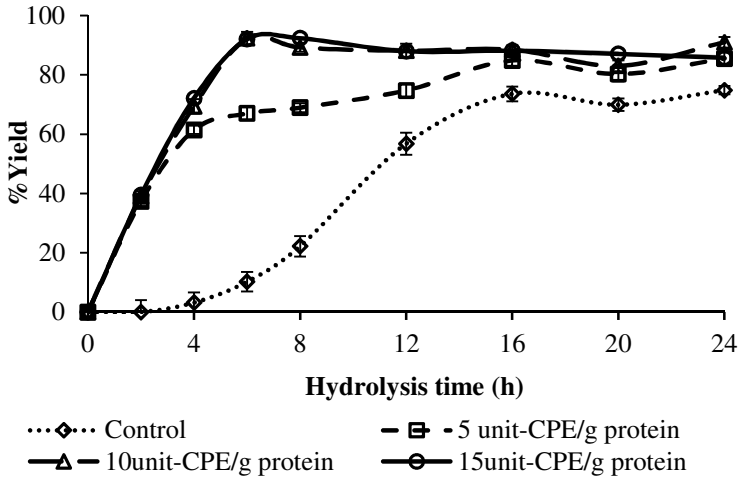

Figure 24 %Yield of virgin coconut oil separated from coconut milk with the aid of CPE at different levels for various times. Bars represent SD (*n=3*).

8.5.3 Comparative study of VCO separation using different separation methods

Table 21 Yield, color in CIE system, lipid oxidation/hydrolysis, moisture content and creaming index of virgin coconut oil separated from coconut milk using different processes.

Parameters	Separation processes		
	Control-6-h	**10-CPE-6-h**	**Fermentation 3 day**
Yield (%)	10.24±3.33^{b}	92.39±2.11^{a}	93.26±2.87^{a}
L^*	100.06±0.01^{a}	100.07±0.02^{a}	100.05±0.03^{a}
a^*	-0.03±0.01^{a}	-0.05±0.01^{a}	-0.04±0.01^{a}
b^*	-0.084±0.03^{a}	-0.086±0.02^{a}	-0.087±0.02^{a}
Peroxide value (mg cumene hydroperoxide/kg oil)	7.31±0.04^{c}	7.51±0.07^{b}	7.75±0.03^{a}
TBARS (mg malonaldehyde/kg oil)	0.19±0.02^{c}	0.34±0.03^{b}	0.97±0.20^{a}
p-anisidine value	2.79±0.96^{b}	4.24±0.90^{b}	27.85±0.55^{a}
Conjugated diene (A_{234})	0.22±0.04^{b}	0.18±0.01^{b}	0.28±0.00^{a}
Free fatty acid content (g/100 g oil)	0.27±0.02^{c}	0.39±0.03^{b}	0.69±0.03^{a}
Moisture content (%)	0.15±0.07^{b}	0.26±0.05^{b}	0.45±0.04^{a}

Means ± SD (n=3).

Different lowercase superscripts ($^{a-c}$) in the same row indicate significant differences ($p<0.05$).

Control-6-h: VCO separated without enzyme for 6 h; 10-CPE-6-h: VCO separated with CPE (10 unit/g protein) for 6 h; Fermentation: VCO separated using the typical fermentation process for 3 days.

8.5.3.1 Yield

Yields of VCO separated from coconut milk with CPE (10 unit/g protein) were compared with that of VCO separated using typical fermentation process (Table 21). The former and the latter had the yields of 92.39±2.11 and 93.26±2.87%, respectively. Yield of VCO separated without CPE (the control) was very low (10.24±3.33%). The use of CPE showed the similar separation efficacy to the fermentation process for VCO separation from coconut milk. Proteases were used to enhance the oil separation from coconut milk (de Moura *et al.*, 2008). Freshly separated coconut milk is a stable emulsion (Raghavendra and Raghavarao, 2010). It is naturally stabilized by coconut proteins such as globulins and albumins as well as phospholipids (Tangsuphoom and Coupland, 2008). Some proteins in the aqueous phase of the coconut milk interact with fat globules and act as emulsifier at the interface (Peamprasart and Chiewchan, 2006).

The production of VCO by enzymatic fermentation includes the usage of yeast, microbe or the other enzymes, which are effective to break down coconut emulsion (Rahayu *et al.*, 2008). The order of effectiveness of protease in improving oil separation yield was found to be as follows: alkaline protease > neutral protease > acid protease (de Moura *et al.*, 2008). Man *et al.* (1996) reported that coconut oil was separated by enzyme mixtures of cellulase, α-amylase, polygalacturonase, and protease (1%, w/w) at pH 7.0 and 60°C with an oil yield of 73.8%. For the control, no CPE was used and the low yield was obtained. Autolysis from microbial enzyme could induce the disruption of fat cells as well as protein denaturation. As a result, the lipids could be released to some extent. Thus, the use of CPE could be a promising means to increase the separation yield of VCO from coconut milk.

8.5.3.2 Color

*L**, *a** and *b** values of VCOs separated from coconut milk without and with CPE (10 unit/g protein) were compared with those of oil separated using typical fermentation process (Table 21). All VCOs were colorless and transparent in nature as evidenced by high *L**-value (100.05±0.03-100.07±0.02). No differences in

*L**, a*, and *b** values of all VCOs separated by different processes were observed (p>0.05). Since the VCO was separated from the fresh coconut meat, there was no pigment associated with deterioration formed and contaminated in oil. VCO heated at excessively high temperatures has slight yellow appearance with a stronger flavor (Fife, 2006). The results suggested the VCO could be separated with the aid of CPE without the negative effect on color.

8.5.3.3 Lipid oxidation and hydrolysis

Lipid oxidation and hydrolysis of VCO separated from coconut milk using different separation processes were monitored as shown in Table 21. VCO produced by the typical fermentation process showed the highest PV (p<0.05), followed by those separated by CPE, and the control (p<0.05). The result suggested that lipid oxidation took place to a higher extent during fermentation for 3 days at room temperature (28-30 °C). Hydroperoxides are the primary products formed by oxidation of the oil (Choe and Min, 2006). The concentration of hydroperoxides generally increases until the advanced stage of oxidation occurs. Formation of hydroperoxide is influenced by heat and some factors such as light, oxygen, metal and fatty acid composition of oil (Choe and Min, 2006). In the present study, the hydrolysis was conducted at 60 °C, which was the optimal temperature for protease in CPE (Senphan and Benjakul, 2012). At high temperature, oxidation of lipid could take place to some degree during incubation for 6 h. Man *et al.* (1996) reported the higher PV in the coconut oil after hydrolysis at 60 °C using the mixture of cellulase, α-amylase, polygalacturonase, and protease, in comparison with control (without enzyme) (Man *et al.*, 1996).

TBARS values of VCOs as influenced by separation methods (Table 21) showed similar results with the PV results. The enhanced oxidation as indicated by higher TBARS was noticeable when coconut milk was subjected to fermentation for 3 days. Hydroperoxides formed at the initial stage could be decomposed to several secondary oxidation products. A larger amount of secondary oxidative products, especially aldehydes, were formed to higher extent during 3 days. Those decomposed products might cause the off-flavor, leading to the poorer oil quality (Senphan and

Benjakul, 2012). The result revealed that autoxidation of fatty acids in coconut oil occurred during separation, especially with the fermentation process.

AnV of VCO varied, depending on the separation processes used. The VCO separated using the fermentation method showed higher AnV than those using CPE and the control ($p<0.05$). The *p*-anisidine reagent reacts with oxidation products, such as aldehydes (principally 2-alkenals and 2, 4-dienals), producing a yellowish product. An increased AnV indicates an increase in the amount of the non-volatile oxidation product (Choe and Min, 2006). Those aldehydes can be further oxidized or participate in dimerization or condensation reactions (Senphan and Benjakul, 2015). It was suggested that the generation of non-volatile secondary product oxidation products occurred during hydrolysis and fermentation. According to Rossell (1989), oils with an anisidine value below 10 were considered as good quality. Since VCO using CPE and control oil had AnV less than 10, it indicated that the quality of those VCOs was relatively good. On the other hand, VCO from the typical fermentation process showed high AnV. The result reconfirmed that the use of CPE was superior to typical fermentation process, in which oil was prone to oxidation.

CD content of VCO separated from coconut milk using different processes is presented in Table 21. VCO from fermentation process showed the highest CD ($p<0.05$). There was no difference in CD between VCO separated by CPE and the control ($p>0.05$). Almost immediately after hydroperoxides are formed, the non-conjugated double bonds that are present in natural unsaturated lipids are converted to conjugated double bonds (Chaijan *et al.*, 2006). Lower CD formation in oil separated using CPE as the processing aid indicated the lower lipid oxidation, in comparison with VCO separated using the typical fermentation process.

FFA content of VCO separated from coconut milk using various processes is shown in Table 21. VCO separated using the typical fermentation process showed higher FFA content than others ($p< 0.05$). An increase in FFA is a well established feature induced by enzymatic hydrolysis of esterified lipids (Lu et al., 2011). Lipase or phospholipase in coconut milk were still active, leading to the enhanced lipid hydrolysis (Elsbach and Rizack, 1963). The hydrolysis might be more enhanced with the extended time of separation. VCO was produced by enzymatic

process generally had low FFA and low water content (0.02 – 0.03 %), clear, good smell and the lifetime might be more than one year (Rahayu *et al.*, 2008). FFAs are responsible for undesirable flavor and aromas in fats. During fermentation of coconut milk, high FFA could be more produced by lipolytic enzymes in the presence of water. Man *et al.* (1997) found that FFAs were high in coconut oils having high moisture content. According to Marina *et al.* (2009), hydrolysis was accelerated by high temperatures and excessive amounts of water. Nevertheless, FFA contents of VCO obtained in this study were relatively low, indicating that the oils were of good quality. Newly produced coconut milk from fresh coconut meat was also used in this study. As a consequence, the lower lipolytic activity was found.

8.5.3.4 Moisture content

Moisture content of VCO separated from coconut milk using different methods is shown in Table 21. VCO separated using typical fermentation process showed higher moisture content (0.45±0.04%) than others (p<0.05). Moisture content in the VCO obtained from enzymatic method and control were found to be 0.15±0.07% and 0.26±0.05%, respectively. Moisture content of the oil is one of the parameters affecting the shelf-life. Higher moisture content could accelerate the hydrolysis process, thereby promoting rancidity (Raghavendra and Raghavarao, 2010). Man *et al.* (1996) reported that free fatty acids were higher in coconut oil having higher moisture content. According to the Asian and Pacific Coconut Community (APCC) standards, good coconut oil must have moisture in the limited range (0.1-0.5%) (APCC, 2003). VCO in the present study had moisture content lower than the limit.

8.5.3.5 Fatty acid profiles

Fatty acid profiles of VCO separated from coconut milk with different separation processes are shown in Table 22. VCO contained medium chain fatty acids (MCFA) as the dominant fatty acids. Lauric acid was the most abundant MCFA (48.21-48.55%). Myristic acid was the second major fatty acid in all VCO samples (19.73-20.68%). Lauric acid content of the control oil was 48.55%. VCO separated

using CPE separation and fermentation method contained lauric acid of 48.44 and 48.21%, respectively. Generally, there was no marked difference in fatty acid profiles between VCO separated with different methods. Palmitic acid ranged from 9.24 to 10.22 g/100g. Olelic aid was found in range of 6.32-6.58 g/100g. The fatty acid composition was comparable to the fatty acid composition guided by the Codex standards (2003) and APCC standards (2003). Since VCO contained a low level of unsaturated fatty acids, it was less susceptible to oxidation.

Table 22 Fatty acid profile of virgin coconut oil separated from coconut milk using different processes

Fatty acids (g/100 g oil)		**Separation processes**		
		Control-6-h*	**10-CPE-6-h**	**Fermentation 3 day**
Caproic acid	C6:0	0.42±0.02†	0.71±0.04	0.14±0.00
Caprylic acid	C8:0	5.91±0.02	5.95±0.12	4.91±0.01
Capric acid	C10:0	5.39±0.01	5.40±0.02	5.03±0.01
Lauric acid	C12:0	48.55±0.01	48.44±0.03	48.21±0.06
Myristic acid	C14:0	19.80±0.00	19.73±0.08	20.68±0.04
Palmitic acid	C16:0	9.28±0.00	9.24±0.05	10.22±0.02
Stearic acid	C18:0	2.96±0.00	2.92±0.02	3.31±0.01
Oleic acid	C18:1 n-9	6.58±0.00	6.51±0.05	6.32±0.03
Linoleic acid	C18:2 n-6	0.95±0.00	0.94±0.01	1.02±0.02
Arachidic acid	C20:0	0.09±0.00	0.09±0.00	0.09±0.00
Unidentified peak		0.08±0.00	0.08±0.00	0.07±0.00

† Means ± SD (n=3).

* Control-6-h: VCO separated without enzyme for 6 h; 10-CPE-6-h: VCO separated with CPE (10 unit/g protein) for 6 h; Fermentation 3 day: VCO separated using the typical fermentation process for 3 days.

8.5.4 Effects of different separation processes on emulsion destabilisation of coconut milk

8.5.4.1 Emulsion stability

Emulsion stability, quantified by creaming index of coconut milk subjected to different treatments, is shown in Table 23. Creaming index provides the indirect information of droplet aggregation (Raghavendra and Raghavarao, 2010). Coconut milk subjected to fermentation showed 47.21% creaming index, which was similar to that found in coconut with treated with CPE (45.45%). The results indicated that oil droplets aggregation took place to a high extent during fermentation or after being treated with CPE. As indicate by higher creaming index, the droplets moved and faster therefore more droplet aggregation occurred when treated with CPE and fermented for a long time. The control coconut milk had creaming index of 34.12%. It indicated that CPE played an important role on destabilization of coconut milk emulsion. Proteins associated with lipid (lipoprotein) emulsions were hydrolyzed by protease, resulting in the liberation of oil, which was subsequently aligned to form the cream (Sant'Anna *et al.*, 2003). Raghavendra and Raghavarao (2010) reported that the coconut milk emulsion treated with and without aspartic protease had creaming index of 49.6 % and 27.7 %, respectively. Thus, CPE could be used to destabilize emulsion of coconut equivalently to the typical fermentation process.

Table 23 Creaming index of coconut milk subjected to different treatments.

Treatments	Creaming index (%)
Control-6-h	34.12 ± 1.38^{b}
10-CPE-6-h	45.45 ± 1.27^{a}
Fermentation 3 day	47.21 ± 2.16^{a}

Means ± SD (n=3).
Different lowercase superscripts ($^{a-c}$) in the same row indicate significant differences ($p<0.05$).
Control-6-h: VCO separated without enzyme for 6 h; 10-CPE-6-h: VCO separated with CPE (10 unit/g protein) for 6 h; Fermentation 3 day: VCO separated using the typical fermentation process for 3 days.

8.5.4.2 Microscopic structure of treated coconut milk

Microscopic structures of coconut milk emulsion subjected to different treatments are illustrated in Figure 25. For untreated coconut milk (control), the oil droplets were in uniform shape as well as size. Droplets were distributed evenly, indicating a stable emulsion (Fig. 25A). After being treated with CPE at room temperature (28-30 °C), the larger oil droplets were observed (Figure 25B). When oil coalesced, loss of spherical shape and large droplets of varying sizes were found. The results clearly indicated the destabilization of emulsion, in which coalescence was found as evidenced by the larger oil droplet. The microscopic structure of coconut milk emulsion subjected to typical fermentation process is shown in Figure 2C. It was observed that the oil droplets coalesced and the larger droplets were formed. The similar result was reported for hydrolysis of coconut milk emulsion by aspartic protease (Raghavendra and Raghavarao, 2010). The use of CPE was therefore found to provide the equivalent efficiency to the typical fermentation process in destabilization of coconut milk emulsion.

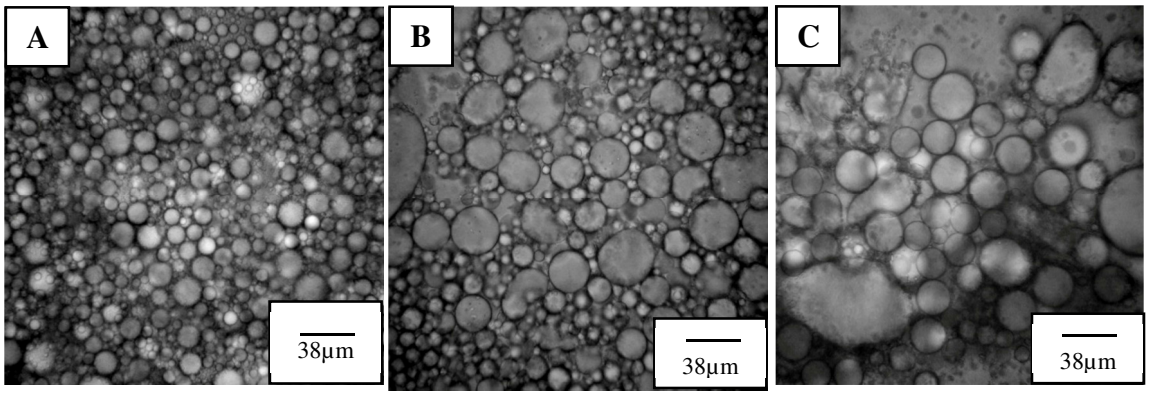

Figure 25 Microscopic structure of coconut milk emulsion treated with different treatments: (A) control; (B) with CPE at 10 unit/g protein and (C) typical fermentation process. Magnification 400×.

8.6. Conclusion

The separation of VCO using CPE (10 unit/g protein) at room temperature (28-30 °C) for 6 h increased the yield without negative effect on fatty

acid composition. VCO had high contents of MCFA, especailly lauric acid and myristic acid. Thus, CPE could be used as promising source of protease for VCO production.

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CHAPTER 9

Comparative study on virgin coconut oil separation using protease from hepatopancreas of Pacific white shrimp and Alcalase

9.1 Abstract

Virgin coconut oil (VCO) was separated from coconut milk with the aid of crude protease extract (CPE) from hepatopancrease of Pacific white shrimp at different levels (5-15 unit/g protein) at 60°C for various hydrolysis times (0-180 min). Yield of VCO increased within the first 90 min (p<0.05)**.** However, Alcalase showed higher efficacy in VCO separation, compared with CPE and the control (without enzyme) (p<0.05). No differences in lipid oxidation of all VCOs separated by different proteases were observed (p>0.05). Alcalase and CPE increased the creaming index and induced the collapse of oil droplets in coconut milk, as determined by the confocal laser scanning microscopy (CLSM) and the phase contrast microscopy. VCOs contained medium chain fatty acid (MCFA), especially lauric acid (C12:0), as the most abundant fatty acid, followed by myristic acid (C14:0). Therefore, VCO could be separated using CPE or Alcalase optimal temperature (60°C) for 90 min, in which the separation could be achieved for a short time without the deterioration induced by oxidation.

9.2 Introduction

Coconut milk is one of the popular cooking ingredients in Thailand, especially for curries and desserts. Coconut milk contains oil and water as the major components (Tansakul and Chaisawang, 2006). Coconut milk is a milky white oil-in-water emulsion and can be obtained by extraction from coconut flesh with or without added water (Seow and Gwee, 1997). The emulsion in coconut milk was naturally stabilized by coconut proteins: globulins and albumins, and phospholipids (Nour *et al.*, 2009; Tansakul and Chaisawang, 2006). Apart from serving as the crucial ingredients, coconut milk can be used as the raw material for virgin coconut oil (VCO) production.

Generally, VCO can be produced from fresh coconut meat, milk, or residue without chemicals or solvents at low temperature without chemical refining, bleaching or de-odorization. Therefore, VCO retains its naturally occurring phyto-chemicals, which contribute to a distinctive coconut taste and smell (Marina *et al.*, 2009). VCO is the purest form of coconut oil and contains natural Vitamin E. It has very low free fatty acid (FFA) content (even without refining) and low peroxide value. VCO contain mainly high lauric acid, a medium chain fatty acid (MCFA), which are resistant to oxidation. The saturated fatty acids in VCO are distinct from animal fats, consisting mainly of long-chain saturated fatty acids (Handayani *et al.*, 2009; Marina *et al.*, 2009). MCFAs are readily utilized by the liver, which leads to greater energy expenditure and enhancement of thermogenesis (Enig, 1996). VCO has a fresh coconut aroma that can be mild to intense, depending on the oil extraction process used (Hamid *et al.*, 2011; Handayani *et al.*, 2009).

VCO can be separated from coconut milk by means of enzymatic hydrolysis, fermentation, refrigeration or mechanical centrifugation (Raghavendra and Raghavarao, 2010). Separation of the oil from the water–oil emulsion can also be accomplished by breaking the emulsion using enzyme or aging for several hours, or by mechanical process using continuous centrifugation (Norulaini *et al.*, 2009). Amongst all processes, the enzyme-assisted separation of coconut oil significantly increased the yield in aqueous system (Tano-Debrah and Ohta, 1997). Enzymatic separation is effective to release the oil by breaking down and destabilization the coconut emulsion (Rahayu *et al.*, 2008). VCO processed by enzymatic separation has more beneficial and safety effect than traditional method from copra, since they are often infected by insects or aflatoxin producing molds associated with the potential toxicity problem during manufacturing (Handayani *et al.*, 2009). Traditional coconut oils are considered to be low quality products, indicated by high moisture and free fatty acid contents (Handayani *et al.*, 2009; Sant'Anna *et al.*, 2003).

Microbial proteases have been widely used for hydrolysis in several industries. However, the cost of enzyme is another concern for user or industry. Thus, the proteases from cheap sources, especially fish processing byproducts, e.g. viscera can be alterative and promising for production of VCO. Shrimp hepatopancreas has been known to contain active proteases, especially trypsin (Sriket *et al.*, 2012).

Hepatopancreas removed from cephalothorax by vacuum sucking machine was reported as the excellent source of proteases (Senphan and Benjakul, 2012). Recently, proteases from shrimp hepatopancreas has shown to increase the yield of VCO from coconut milk, when the separation was carried out at room temperature (28-30°C). However, it took around 6 h for separation (Senphan and Benjakul, 2015a). Additionally, the separation for a long time might induce the deterioration associated with lipid oxidation. Therefore, the use of protease at the optimal temperature might enhance the separation efficacy for VCO, in which the shorter time was required. The present study aimed to comparatively investigate the uses of crude protease extract from Pacific white shrimp hepatopancreas and Alcalase at optimal temperature (60°C) on separation efficiency and properties of VCO from coconut milk.

9.3 Objective

To separate virgin coconut oil from coconut oil from coconut milk using protease from Pacific white shrimp hepatopancreas

9.4 Materials and methods

9.4.1 Chemicals

Palmitic acid, cupric acetate, *p*-anisidine, ammonium thiocyanate, cupric acetate and pyridine were purchased from Sigma (St. Louis. MO, USA). Trichloroacetic acid, anhydrous sodium sulfate, isooctane and ferrous chloride were obtained from Merck (Darmstadt, Germany). 2-Thiobarbituric acid and 1,1,3,3-tetramethoxypropane were purchased from Fluka (Buchs, Switzerland). Methanol, ethanol, chloroform, petroleum ether, hydrochloric acid, sulfuric acid and ammonium thiocyanate were procured from Lab-Scan (Bangkok, Thailand). Alcalase (2.4L) with the activity of 2.4 Au/g and a density of 1.17 g/mL was obtained from East Asiatic Co. (Thailand) Ltd. (Bangkok, Thailand).

9.4.2 Preparation of coconut milk

Fresh coconut milk from ‘ordinary tall’ cultivar (12–14 months’ maturity) was purchased from a local market in Hat Yai, Songkhla province,

Thailand. The mature coconuts were used for coconut milk preparation. Coconuts were subjected to deshelling, paring and removal of water. The white coconut kernel was grated using a rotary wedge cutter (Motor15, BSHatyai, Songkhla, Thailand). Coconut milk was prepared by pressing the grated coconut using a hydraulic press (Model stainless steel hydraulic press A2, Sakaya, Bangkok, Thailand). Coconut milk obtained was transported to the Department of Food Technology, Prince of Songkla University, Hat Yai, Songkhla and immediately used for VCO separation. Coconut milk contained 32.11% fat as determinate by the Gerber method (AOAC, 2000a).

9.4.3 Preparation of crude protease extract from hepatopancreas

Fresh hepatopancreas of Pacific white shrimp was collected from Sea wealth frozen food Co., Ltd., Songkhla province, Thailand. Hepatopancreas was packaged in polyethylene bag, stored in ice using a sample/ice ratio of 1:2 (w/w) and transported to the Department of Food Technology, Prince of Songkla University within 2 h. Upon arrival, the sample was cut into pieces with a thickness of 1–1.5 cm. Sample was ground into powder in the liquid nitrogen using a National Model MX-T2GN blender (Taipei, Taiwan) and homogenized in three volumes of acetone to remove fat using a homogenizer (model PT-MR2100, POLYTRON®, KINEMATICA AG, Littau/Luzern, Switzerland) at 15,000 rpm at -20°C for 2 min according to the method of Kishimura and Hayashi (2002). The homogenate was stirred continuously using a magnetic stirrer model BIG SQUID (IKA®-Werke GmbH & CO.KG, Staufen, Germany) at 4°C for 30 min and filtered in vacuo on Whatman No. 4 filter paper (Whatman International Ltd., Maidstone, England). The residue obtained was then homogenized in two volumes of acetone (-20°C) for 2 min and stirred at 4°C for another 30 min. The homogenate was filtered as described above. Then the residue was left at room temperature until dried and free of acetone odor.

To prepare the crude protease extract, acetone powder was suspended in an extraction buffer (10 mM Tris–HCl, pH 8.0 containing 1 mM $CaCl_2$) at a ratio of 1:50 (w/v) and stirred at 4°C for 3 h. The suspension was centrifuged for 10 min at 4°C at 10,000 ×g to remove the tissue debris using a refrigerated centrifuge (Beckman Coulter, Avanti J-E Centrifuge, Fullerton, CA, USA). The supernatant was lyophilised using a SCANVAC CoolSafe™ freeze-dryer (CoolSafe 55, ScanLaf A/S,

Lynge, Denmark). The powder was referred to as “crude protease extract, CPE”. Before use, the lyophilized sample was dissolved with cold distilled water (4°C) at a ratio of 1:5 (w/v). The solution was determined for protease activity and further used.

9.4.4 Protease activity assay

Protease activity of CPE and Alcalase was measured using casein as a substrate according to the method of An *et al.* (1994). The assay was performed at pH 8 using 50 mM Tris–HCl buffer containing 10 mM $CaCl_2$ and 60°C. One unit of activity was defined as that releasing 1 μmol of tyrosine equivalent per min (μmol Tyr equivalent /min).

9.4.5 Effect of CPE at different levels on virgin coconut oil separation

Enzymatic separation of VCO from coconut milk was performed according to the method of Raghavendra and Raghavarao (2010) with a slight modification. The coconut milk (100 mL) in a 250 mL-Erlenmeyer flask was adjusted to pH 8 using 2 M NaOH. Therefore, CPE was added into coconut milk to obtain different protease levels (5 10 and 15 units/g protein). The hydrolysis was allowed to proceed at optimal temperature of CPE (60°C) for different times (0, 10, 20, 30, 45, 60, 90, 120 and 180 min). At the time designated, the mixtures were cooled using in water (25°C) for approximately 10 min. Subsequently, the mixtures were transferred into the 200 mL tube and centrifuged at 4,900×g at 25°C for 30 min using Allegra^{TM} 25R centrifuge (Beckman Coulter, Palo Alto, CA, USA). Upper layer rich in oil was then collected using a pasteur pipette. The bottom (residue) and the middle (emulsion) phases were discarded. The collected oil was placed in a vial, flushed with nitrogen gas, sealed tightly and kept at -40°C until analysis.

9.4.5.1 Yield

The yield of VCO was calculated and expressed as the percentage of oil separated, relative to total oil content in coconut milk as determined by the Gerber method (AOAC, 2000a).

9.4.5.2 SDS–polyacrylamide gel electrophoresis (SDS–PAGE)

Protein patterns of coconut milk at various hydrolysis times were determined according to the method of Laemmli (1970) using 4% stacking gel and 10% separating gel. Coconut milk with different hydrolysis conditions (10 mL) was homogenized with 10 mL of 5% SDS at a speed of 12,000 rpm for 1 min. The homogenate was heated at 85°C for 1 h, followed by centrifugation at 7500g for 10 min at 25°C using a centrifuge (Sorvall, Model RC-B Plus, Newtown, CT, USA). The protein concentration of supernatant was determined by the Biuret method (Robinson and Hogden, 1940), using bovine serum albumin (BSA) as standard. The samples were mixed at 1:1 (v/v) ratio with the SDS–PAGE sample buffer (0.125 M Tris–HCl, pH 6.8; 4% SDS; 20% glycerol; 10% β-mercaptoethanol) and boiled for 5 min. The samples (10 μg) were loaded onto the gel made of 4% stacking and 10% separating gels. Electrophoresis was performed using a vertical gel electrophoresis unit (Mini-protein II; Bio-Rad Laboratories, Richmond, CA, USA) at the constant voltage of 200 V/plate. The gels were stained with Coomassie Brilliant Blue R-125 (0.125%) in 25% methanol and 10% acetic acid. Destaining was performed using 40% methanol and 10% acetic acid. High and low molecular weight (MW) markers were used for estimation MW of proteins.

9.4.6 Comparative study of VCO separation using different protease

VCO separation was carried out using CPE (10 units/g protein) or Alcalase (10 units/g proteins). The hydrolysis was performed at pH 8 and 60°C for 90 min. The control was prepared in the same manner, except that either CPE or Alcalase was excluded. All oil samples were collected and stored at -40°C as previously described. All samples were calculated for yield and subjected to analyses.

9.4.7 Analyses

9.4.7.1 Color determination

Color of VCO was measured using a colorimeter (HunterLab, Model colorFlex, VA, USA). The color was reported in CIE system. $L*$, $a*$ and $b*$-values indicate lightness, redness/greenness and yellowness/blueness, respectively.

9.4.7.2 Measurement of peroxide value (PV)

PV was determined using the ferric thiocyanate method (Chaijan *et al.*, 2006). To 50 µL of VCO sample (10-fold dilution using 75% ethanol, v/v), 2.35 mL of 75% ethanol (v/v), 5 0 µL of 30% ammonium thiocyanate (w/v) and 50 µl of 20 mM ferrous chloride solution in 3.5% HCl (w/v) were added and mixed thoroughly. After 3 min, the absorbance of the colored solution was read at 500 nm using a UV-1601 spectrophotometer (Shimadzu, Kyoto, Japan). The blank was prepared in the same manner, except the distilled water was used instead of ferrous chloride. PV was calculated after blank subtraction. A standard curve was prepared using cumene hydroperoxide with the concentration range of 0.5–2 ppm. PV was expressed as mg cumene hydroperoxide/kg oil.

9.4.7.3 Measurement of thiobarbituric acid-reactive substances (TBARS)

TBARS were determined as described by Buege and Aust (1978). VCO sample (0.5 g) 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 ºC) for 10 min to develop a pink color, cooled with running water and centrifuged at 3600×*g* at 25 ºC for 20 min. 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 value was calculated and expressed as mg malonaldehyde/kg oil.

9.4.7.4 Measurement of *p*-anisidine value (AnV)

AnV of VCO sample was analyzed according to the method of AOCS (AOCS, 1990). VCO sample (100 mg) was dissolved in 25 mL of isooctane. The solution (2.5 mL) was mixed with 0.5 mL of 0.5% (w/v) *p*-anisidine in acetic acid for 10 min. The absorbance was read at 350 nm using a spectrophotometer. AnV value was calculated using the following formula:

$$AnV = 25 \times \left(\frac{(1.2 \times A_2) - A_1}{W} \right)$$

where A_1 and A_2: the absorbance at 350 nm before and after adding *p*-anisidine, respectively; W= weight of oil sample (g)

9.4.7.5 Determination of conjugated diene (CD)

CD formed in the sample was measured as per to the method of Frankel *et al.* (1997). VCO sample (0.1 mL) was dissolved in methanol (5.0 mL) and the absorbance at 234 was read using a spectrophotometer. Content of conjugated diene was expressed as the increase in absorbance at 234 nm.

9.4.7.6 Determination of saponification number (SV) and iodine value (IV)

Saponification number and iodine value were determined as per the IUPAC (1974) method and expressed as mg KOH/g oil and g I_2/100g oil, respectively.

9.4.7.7 Measurement of free fatty acid

Free fatty acid (FFA) content was determined following the method of Lowry and Tinsley (1976). VCO sample (0.1 g) was added with 5 mL of isooctane and swirled vigorously to dissolve the sample. The mixture was then treated with 1 mL of 5% (w/v) cupric acetate-pyridine reagent, prepared by dissolving 5 g of the reagent grade cupric acetate in 100 mL of water, filtering and adjusting the pH to 6.0-6.2 using pyridine. The mixture was shaken vigorously for 90 sec using a Vortex-Genie2 mixer (Bohemia, NY, USA) and allowed to stand for 20 sec. The upper layer was subjected to absorbance measurement at 715 nm. A standard curve was prepared

using palmitic acid in isooctane at concentrations ranging from 0 to 10 μmol/ mL. FFA content was expressed as g FFA/100 g oil.

9.4.8.8 Determination of moisture content

VCO sample was analysed for moisture content according to the method of AOAC (2000b)

9.4.9 Determination of fatty acid profile

Fatty acid profile of VCO samples was determined as fatty acid methyl esters (FAMEs). FAMEs were prepared according to the method of AOAC (2000). The prepared FAMEs were injected to the gas chromatography (Shimadzu, Kyoto, Japan) equipped with the flame ionization detector (FID) at a split ratio of 1:20. A fused silica capillary column (30 m×0.25 mm), coated with bonded polyglycol liquid phase, was used. The analytical conditions were: injection port temperature of 250°C and detector temperature of 270 ºC. The oven was programmed from 170 to 225 ºC at a rate of 1 ºC /min (no initial or final hold). Retention times of FAME standards were used to identify chromatographic peaks of the samples. Fatty acid content was calculated, based on the peak area ratio and expressed as g fatty acid/100 g oil.

9.4.10 Effect of different proteases on emulsion destabilization of coconut milk

Coconut milks treated with CPE or Alcalase at a level of 10 units/g protein at pH 8 and 60°C for 90 min were prepared. The treated samples were determinated for emulsion stability, particle size distribution and microstructure of oil droplets. Coconut milk without protease treatment was also prepared and used as the control.

9.4.10.1 Measurements of creaming index

Creaming index, an indicator of emulsion stability, was measured according to the method of Raghavendra and Raghavarao (2010) with a slight modification. The samples were allowed to stand for 6 h at room temperature (28-

30°C) to allow the separation into the cream (top) and the aqueous (bottom) phases. Total height of the emulsion in the test tube (HE) and the height of the aqueous layer (HS) were measured. The extent of creaming was expressed as creaming index, which was calculated using the following formula:

$$\text{Creaming index } (\%) = \frac{\text{HS}}{\text{HE}} \times 100$$

9.4.10.2 Determination of particle size

Particle size distribution of emulsions was determined using a laser particle size analyzer (LPSA) (Model LS 230, Beckman Coulter®, Fullerton, CA, USA) as per the method of Castellani, Belhomme, David-Briand, Guérin-Dubiard, and Anton (2006). The surface-weighted mean particle diameter (d_{32}) and the volume-weighted mean particle diameter (d_{43}) of the emulsion droplets were measured.

9.4.10.3 Determination of microstructure of oil droplets

9.4.10.3.1 Confocal laser scanning microscopy (CLSM)

The microstructures of coconut milk samples were examined with a confocal laser scanning microscope (CLSM) (Model FV300; Olympus, Tokyo, Japan). The samples were dissolved in Nile blue A solution (1:10) and manually stirred until uniformity was achieved. Fifty microliters of sample solutions 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 (HeNe-G) for protein analysis. A magnification of 400× was used.

9.4.10.3.2 Phase contrast microscopy

Oil droplets in coconut milk samples were observed under phase contrast microscope (Model FV300; Olympus, Tokyo, Japan) equipped with camera. Samples were placed on a glass slide, covered with cover slip and observed at 400× magnification.

9.4.11 Statistical analysis

Experiments were run in triplicate using three different lots of samples. Data were subjected to analysis of variance (ANOVA). Comparison of means was carried out by Duncan's multiple range test. For pair comparison, T-test was used (Steel and Torrie, 1980). Statistical analysis was performed using the Statistical Package for Social Science (SPSS 11.0 for windows, SPSS Inc., Chicago, IL, USA).

9.5. Results and discussion

9.5.1 Effect of CPE levels and hydrolysis time on yield of VCO from coconut milk

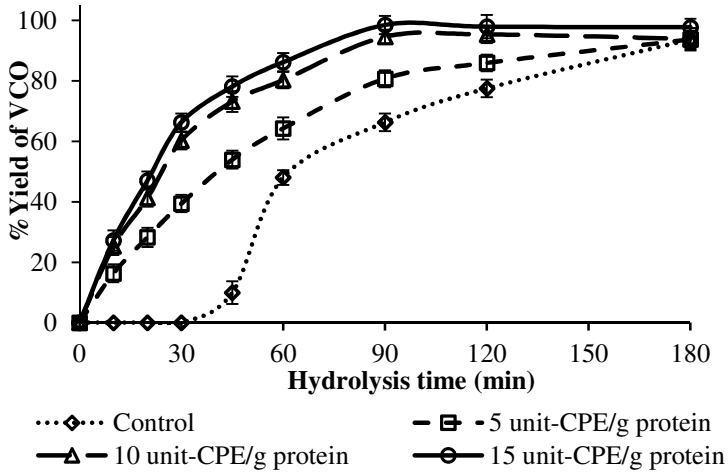

Figure 26 %Yield of virgin coconut oil separated from coconut milk with the aid of crude protease extract from Pacific white shrimp hepatopancreas at different levels at 60 ºC for various times. Bars represent SD (n=3).

Effect of CPE at different levels (5, 10 and 15 unit/g protein) and various hydrolysis times on the yield of VCO from coconut milk is shown in Figure 26. At the same hydrolysis time, the yield of VCO increased with increasing protease levels (p<0.05). Within the first 90 min of hydrolysis, the increase in VCO yield was observed for the coconut milk treated with CPE at levels of 10 and 15 unit/g protein as the hydrolysis time increased. With lower amount of CPE (5 unit/g protein) used, the yield increased continuously as hydrolysis time increased up to 180 min (p<0.05). In general, no differences in VCO yield were noticeable when protease levels of 10

and 15 (units/g protein) were used (p>0.05). Therefore, the addition of CPE from Pacific white shrimp at a sufficient amount was effective in increasing the yield of VCO from coconut milk. Excessive amount of protease did not enhance the separation efficacy. During the incubation of coconut milk at 60°C for longer time, the heat might induce the denaturation of proteins in coconut milk, which played a role in stabilizing the emulsion. As a result, the emulsion was collapsed as indicated by the increased amount of oil liberated. However, no oil separation from the coconut milk was obtained without CPE addition when the time was less than 30 min. VCO could be liberated gradually after 30 min and the highest yield (93.68%) was obtained when the coconut milk was allowed to stand at 60°C for 180 min. This typical curve was also reported by Senphan and Benjakul (2015a) when CPE was used for separation of VCO from coconut milk at room temperature (28-30°C). Furthermore, the increase in CPE levels (5–15 units/g protein) resulted in an increase in yields. VCO separated using CPE (10 unit/g protein) at room temperature for 6 h had the highest yield of 92.39% (Senphan and Benjakul, 2015a). CPE was reported to exhibit the excellent hydrolytic activity towards proteins (Senphan and Benjakul, 2012). CPE was able to assist in hydrolysis of proteins stabilizing emulsion in coconut milk. As a consequence, the oil could be released within the short time. At 60°C, protease in CPE exhibited the highest activity (Senphan and Benjakul, 2012). The similar phenomenon was reported for coconut milk emulsion subjected to hydrolysis by commercial protease (Raghavendra and Raghavarao, 2010). A maximum yield of 95.3 % was obtained when coconut milk was treated with aspartic protease at concentration of 0.02 mg/g (Raghavendra and Raghavarao, 2010). Thus, VCO could be separated from coconut milk with the aid of CPE at an appropriate level (10 unit/g protein) at 60°C within 90 min. Two-fold longer time was used for the control, incubated for 180 min at 60°C.

9.5.2 Hydrolysis of coconut milk proteins by CPE from hepatopancreas of Pacific white shrimp

Protein patterns of coconut milk protein hydrolysed by CPE (10 unit/g protein) from the hepatopancreas of Pacific white shrimp during the incubation at 60°C for 0–180 min are shown in Figure 27. Coconut milk consisted of protein with

MW of 56 kDa as the dominant protein. Additionally, proteins with MW of 31, 25, 19, 17 and 16 kDa were also found in coconut milk. Proteins with MW less than 19 kDa were also present in coconut milk. Marked degradation of proteins with MW of 56 and 16 kDa was observed after 10 min of hydrolysis and the complete hydrolysis was noticed after 20 min with the concomitant occurrence of degradation products having very low MW. Protein with MW of 56 kDa was more likely cocosin (Tangsuphoom and Coupland, 2008). The major protein (~65%) in coconut endosperm is 11S globulin known as cocosin (Garcia et al., 2005), a hexamer of 55 kDa subunits with each subunit comprising an acidic (32–34 kDa) and basic (22–24 kDa) polypeptides linked by a disulfide bridge (Garcia et al., 2005; Tangsuphoom and Coupland, 2008). Cocosin is believed to play a more important role in governing the stability of coconut milk than either the albumin or the 7S globulin fraction (Tangsuphoom and Coupland, 2008). The result suggested that cocosin was susceptible to hydrolysis by protease from Pacific white shrimp hepatopancreas. Tangsuphoom and Coupland (2008) reported that the protein bands of coconut milk at 26 and 18 kDa were identified as the albumin fraction. The result indicated that protein with Mw of 56 and 16 kDa were susceptible to hydrolysis induced by protease in hepatopancreas of Pacific white shrimp. Those proteins might play a role as the emulsifier in stabilizing the coconut oil in coconut milk emulsion. After those proteins were hydrolysed, emulsion was not stable with concomitant release of oil from coconut milk.

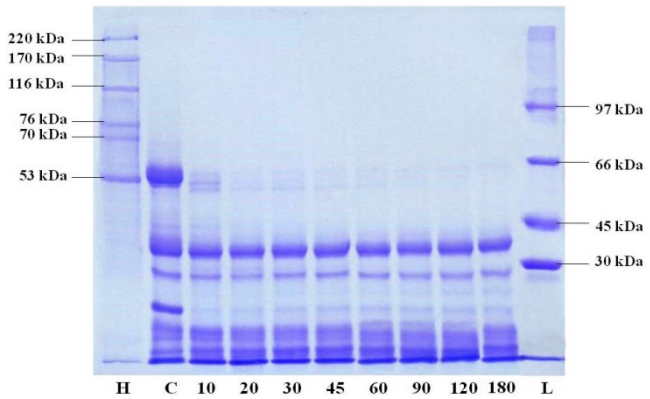

Figure 27 SDS-PAGE patterns of coconut milk proteins during hydrolysis by crude protease extract from Pacific white shrimp hepatopancreas at 10 unit/g protein. Numbers designate the hydrolysis time (min). H: high molecular weight standards; C: control (coconut milk protein without protease); L: low molecular weight standards.

9.5.3 Chemical composition and quality of VCO

Table 24 Yield, color and chemical compositions of virgin coconut oil separated with the aid of crude protease extract from Pacific white shrimp hepatopancreas or Alcalase.

Parameters	Treatments		
	Control-90-min	**10-CPE-90-min**	**10-Alc-90-min**
Yield (%)	69.60±3.01^{c}	93.83±1.38^{b}	98.25±1.27^{a}
*L**	100.02±0.02^{a}	100.03±0.03^{a}	100.06±0.05^{a}
*a**	-0.07±0.02^{a}	-0.08±0.03^{a}	-0.07±0.01^{a}
*b**	-0.091±0.06^{a}	-0.082±0.05^{a}	-0.087±0.04^{a}
Moisture content (%)	0.03±0.01^{a}	0.04±0.02^{a}	0.04±0.01^{a}
Peroxide value (mg cumene hydroperoxide /kg oil)	2.06±0.32^{b}	7.77±0.83^{a}	8.28±0.51^{a}
TBARS (mg malonaldehyde/kg oil)	0.19±0.02^{a}	0.17±0.03^{a}	0.18±0.05^{a}
p-anisidine value	1.52±0.21^{a}	1.79±0.55^{a}	1.93±0.38^{a}
Conjugated diene (A_{234})	0.71±0.10^{a}	0.69±0.04^{a}	0.54±0.12^{a}
Free fatty acid content (g/100 g oil)	0.11±0.01^{c}	0.26±0.03^{a}	0.19±0.02^{b}
Saponification value (mg KOH/g oil)	255.65±1.57^{a}	254.21±1.15^{a}	255.83±1.37^{a}
Iodine value (g I_2/100g oil)	4.81±0.28^{a}	4.89±0.74^{a}	4.74±0.43^{a}

Means ± SD (n=3).

Different lowercase superscripts in the same column indicate significant differences (p<0.05). Control-90-min: control VCO (without protease and incubated at 60°C for 90 min); 10-CPE-90-min: VCO separated with CPE (10 unit/g protein) at 60°C for 90 min; 10-Alcalase-90-min: VCO separated with Alcalase (10 unit/g protein) at 60°C for 90 min.

9.5.3.1 Yield

Yields of VCO separated from coconut milk treated with CPE (10 unit/g protein) were compared with those of VCO prepared using Alcalase (10 unit/g protein) (Table 24). The same condition (60°C, 90 min) was used for both proteases. The yields were 93.83±1.38 and 98.25±1.27%, respectively. It was noted that Alcalase at the same level (10 unit/g protein) showed higher efficacy in separating oil

from coconut milk. Yield of VCO separated without protease (control) was 69.60±3.01%. The use of CPE or Alcalase for VCO separation from coconut milk resulted in a higher yield, compared with the control (p<0.05). VCO separated using CPE (10 unit/g protein) at room temperature (28-30°C) for 6 h and using typical fermentation process for 3 days had the yield of 92.39 and 93.26%, respectively (Senphan and Benjakul, 2015a). Proteases were used to enhance the oil separation from coconut milk (de Moura et al., 2008). Coconut milk is an oil-in-water emulsion, stabilised by the naturally occurring proteins (albumins and globulins) and phospholipids (lecithin and cephalin) (Raghavendra and Raghavarao, 2010). Some proteins in the aqueous phase is believed to play more important role in governing the stability of coconut milk by interacting with fat globules and acting as emulsifier at the interface (Peamprasart and Chiewchan, 2006). The basic principal of enzymatically coconut oil production is to make the emulsion becoming unstable via cleavage or coagulation of protein surrounding oil droplets. As a result, oil could be released from the emulsion (Handayani et al., 2009; Rahayu et al., 2008). Nevertheless, yield of oil was different when different proteases were used. Activities of enzymes were affected by substrate and enzyme concentration, pH, temperature, and incubation time (Handayani et al., 2009). Man et al. (1996) studied the use an enzyme mixture at 1% (w/w) each of cellulase, α-amylase, polygalacturonase, and protease at pH 7.0 and a separation temperature of 60°C for coconut oil separation. The recovery of 73.8% and good-quality oil were achieved. For the control, no CPE or Alcalase was used and the lower yield was obtained. During incubation at 60 C° for an appropriate time, the disruption of fat cells as well as protein denaturation could occur to some degree. This resulted in the collapse of emulsion, in which oil could be separated from coconut milk. Thus, the use of CPE or Alcalase could be a promising means to increase the separation yield of VCO from coconut milk.

9.5.3.2 Color

L^*, a^* and b^* values of VCO separated from coconut milk with CPE (10 unit/g protein) or Alcalase (10 unit/g protein) in comparison with the control are shown in Table 24. All VCOs were colorless and transparent in nature as evidenced by high L^*-value (100.02±0.02-100.06±0.05). No differences in L^*, a^*, and b^* values

of all VCOs separated under different conditions were observed (p>0.05). Since the VCO was separated from the fresh coconut meat, no pigments associated with deterioration formed were contaminated in oil. VCO was found to be almost colorless, whilst the refining, bleaching, and deodorizing (RBD) coconut oil was described as having a distinct yellow color (Marina et al., 2009). VCO heated at excessively high temperatures has slight yellow appearance with a stronger flavor (Fife, 2006). The results suggested that VCO could be separated with the aid of CPE or Alcalase without the negative effect on color.

9.5.3.3 Chemical composition and quality of VCO

9.5.3.1 Moisture content

Moisture content of VCO separated from coconut milk using different proteases in comparison with the control is shown in Table 24. No differences in moisture content were observed between all VCO samples (p>0.05). Moisture content in all VCO was 0.03-0.04% which is lower than VCO separated using CPE (10 unit/g protein) at room temperature (28-30°C) for 6 h (0.26%) (Senphan and Benjakul, 2015a). Moisture content of the oil is one of the parameters affecting the shelf-life. Higher moisture content could accelerate the hydrolysis process, thereby promoting rancidity (Raghavendra and Raghavarao, 2010). Man et al. (1996) reported that free fatty acids were higher in coconut oil with higher moisture content. According to the Asian and Pacific Coconut Community (APCC) standards, good coconut oil must have moisture in the limited range (0.1-0.5%) (APCC, 2003). VCO in the present study had lower moisture content than the limit. This reflected the presence of lipophilic compounds in VCO, particularly triglyceride, which showed low capacity of binding water or moisture during separation.

9.5.3.2 Fatty acid profiles

Fatty acid profiles of VCO separated from coconut milk without and with different proteases are shown in Table 25. Lauric acid was the most abundant medium chain fatty acid (MCFA) (50.66-51.06%). Myristic acid was the second major fatty acid in all VCO samples (18.20-18.47%). Lauric acid content of the

control oil was 50.66%. VCO separated using CPE and Alcalase contained lauric acid at 51.06 and 50.69%, respectively. The most abundant MCFA found in coconut oil is lauric acid, which is responsible for many of coconut oil's health benefits (Khoramnia *et al.*, 2013). MCFAs have 6 to 12 carbons, and include caproic (C6:0), caprylic (C8:0), capric (C10:0), and lauric (C12:0) acids (Norulaini *et al.*, 2009). Generally, there was no marked difference in fatty acid profile between VCO separated with different proteases as well as the control. Palmitic acid ranged from 8.24 to 8.44 g/100g. Olelic aid was found in range of 5.30-5.45 g/100g. Fatty acid composition of VCO in the present study was comparable to that composition guided by the Codex standards (2003) and APCC standards (2003). Since VCO had the low level of unsaturated fatty acids, it was less susceptible to oxidation.

Table 25 Fatty acid profile of virgin coconut oil separated from coconut milk with the aid of crude protease extract from Pacific white shrimp hepatopancreas or Alcalase.

Fatty acids (g/100 g oil)		Treatments		
		Control-90-min*	10-CPE-90-min	10-Alc-90-min
Caproic acid	C6:0	0.43±0.00†	0.24±0.00	0.41±0.02
Caprylic acid	C8:0	6.65±0.63	6.27±0.78	6.22±0.25
Capric acid	C10:0	6.08±0.11	6.01±0.15	6.00±0.01
Lauric acid	C12:0	50.66±0.36	51.06±0.47	50.69±0.28
Tridecanoic acid	C13:0	0.03±0.00	0.02±0.00	0.03±0.01
Myristic acid	C14:0	18.20±0.24	18.47±0.47	18.47±0.09
Palmitic acid	C16:0	8.24±0.11	8.34±0.25	8.44±0.04
Stearic acid	C18:0	2.74±0.03	2.80±0.09	2.78±0.01
Cis-9-Octadecadienoic acid	C18:1 n-9	5.30±0.06	5.31±0.17	5.45±0.02
Cis-9,12-Octadecadienoic acid	C18:2 n-6	0.97±0.01	0.96±0.03	1.04±0.00
Arachidic acid	C20:0	0.06±0.00	0.06±0.00	0.06±0.00
Cis-11-Eicosenoic acid	C20:1 n-9	0.03±0.00	0.03±0.00	0.03±0.00
Unidentified peak		0.60±0.09	0.43±0.01	0.39±0.01

† Means ± SD (n=3).

* Control-90-min: control VCO (without protease and incubated at 60°C for 90 min); 10-CPE-90-min: VCO separated with CPE (10 unit/g protein) at 60°C for 90 min; 10-Alcalase-90-min: VCO separated with Alcalase (10 unit/g protein) at 60°C for 90 min.

9.5.3.3 Lipid oxidation and hydrolysis

Lipid oxidation and hydrolysis of VCO separated from coconut milk with different separation conditions were monitored as shown in Table 24. PV of VCO separated from coconut milk using CPE or Alcalase and the control were 7.77±0.83, 8.28±0.51 and 2.06±0.32 mg cumene hydroperoxide /kg oil, respectively. VCO separated using CPE or Alcalase showed higher PV than the control (p<0.05).VCO separated by CPE (10 unit/g protein) at room temperature (28-30°C) for 6 h had PV of 7.51±0.07mg cumene hydroperoxide /kg oil (Senphan and Benjakul, 2015a). The result suggested that lipid oxidation took place to a higher extent during hydrolysis by proteases for 90 min at 60°C. Hydroperoxides are the primary products formed via the oxidation of oil (Choe and Min, 2006). During hydrolysis of proteins, especially those with MW of 56 and 26 kDa (Figure 27), the mineral or metals associated with those proteins could be released and acted as pro-oxidants in coconut milk. Those pro-oxidants could induce the lipid oxidation as evidenced by the higher PV. The concentration of hydroperoxides generally increases until the advanced stage of oxidation occurs. Formation of hydroperoxide is influenced by heat and some factors such as light, oxygen, metal and fatty acid composition of oil (Choe and Min, 2006). In the present study, the hydrolysis was conducted at 60°C, which was the optimal temperature for protease in CPE and Alcalase (Senphan and Benjakul, 2012). At high temperature, oxidation of lipid could take place to some degree during incubation for 90 min. This was indicated by PV of 2.06 mg cumene hydroperoxide /kg oil found in the control oil. Raghavendra and Raghavarao (2011) also reported the higher PV in the coconut oil separated from coconut milk after hydrolysis at 60°C using aspartic protease at a concentration of 0.02 mg/g, followed by chilling (5°C) and thawing when compared with the commercial coconut oil samples (Raghavendra and Raghavarao, 2011).

TBARS values of VCOs as influenced by protease treatments are shown in Table 24. No differences in TBARS were observed between all VCO samples (p>0.05). When lipid oxidation occurs, unstable hydroperoxide is formed at the initial stage and is decomposed readily to several secondary oxidation products. Those final products can be detected as TBARS (Chaijan et al., 2006). Those products

might cause the off-flavor and odor, leading to the poorer oil quality (Chaijan et al., 2006; Senphan and Benjakul, 2012). Although PV of VCO was different amongst the different samples, there was no difference in TBARS. Low TBARS suggested that most hydroperoxides formed were not decomposed to the secondary products. Furthermore, those low MW volatiles might be lost, as indicated by low TBARS values.

VCO separated using the CPE or Alcalase showed similar AnV to the control oil (p>0.05). The similar result was noticeable, in comparison with TBARS values. AnV of VCO separated from coconut milk using CPE or Alcalase and the control were 1.79±0.55, 1.93±0.38 and 1.52±0.21, respectively. However, VCO separated using CPE (10 unit/g protein) at room temperature (28-30°C) for 6 h had AnV of 4.24±0.90 (Senphan and Benjakul, 2015a). The *p*-anisidine reagent reacts with oxidation products, such as aldehydes (principally 2-alkenals and 2, 4-dienals), producing a yellowish product. An increased AnV indicates an increase in the amount of the non-volatile oxidation product (Choe and Min, 2006). Those aldehydes can be further oxidised or participate in dimerisation or condensation reactions (Senphan and Benjakul, 2015b). The resulted suggested that the generation of non-volatile secondary product oxidation products occurred during VCO separation. According to Rossell (1989), oils with an AnV below 10 were considered as good quality. Since VCO using CPE, Alcalase and control oil had AnV less than 10, it indicated that the quality of those VCOs was relatively good.

CD content of VCO separated from coconut milk using different conditions is presented in Table 24. No differences in CD were observed between all VCO samples (p>0.05). Almost immediately after hydroperoxides are formed, the non-conjugated double bonds that are present in natural unsaturated lipids are converted to conjugated double bonds (Chaijan *et al.*, 2006). Low CD formation in all VCO samples, regardless of protease treatment, indicated the low lipid oxidation taken place in VCO. This was due to the abundance of MCFAs, which were less prone to oxidation.

FFA content of VCO separated from coconut milk using various proteases is shown in Table 24. VCO separated using CPE showed the highest FFA

content ($p<0.05$), followed by those separated by Alcalase, and the control (0.26±0.03, 0.19±0.02 and 0.11±0.01 g/100 g oil, receptively) ($p<0.05$). Senphan and Benjakul (2015a) reported that VCO separated using CPE (10 unit/g protein) at room temperature (28-30°C) for 6 h had the higher FFA of 0.39±0.03 g/100 g oil. An increase in FFA (lipolysis) is a well established feature in coconut milk, resulting from the enzymatic hydrolysis of esterified lipids. Lipase or phospholipase in coconut milk were still active, leading to the enhanced lipid hydrolysis (Elsbach and Rizack, 1963). FFA is responsible for undesirable flavor in oils and fats. FFA is formed during hydrolytic rancidity, which is the hydrolysis of an ester by lipase or moisture (Raghavendra and Raghavarao, 2010). The hydrolysis might be more enhanced with the extended time and temperature of separation. The VCO was produced by enzymatic process contained low FFA. It had visual appearance of water clear, good smell and long lifetime (Rahayu et al., 2008). Man et al. (1997) found that FFAs were high in coconut oils having high moisture content. According to Marina et al. (2009), hydrolysis was accelerated by high temperatures and excessive amounts of water. Nevertheless, FFA contents of VCO obtained in this study were relatively low, indicating that the oils were of good quality. Newly produced coconut milk from fresh coconut meat was also used in this study. As a consequence, the lower lipolysis occurred.

9.5.3.4 SV and IV

SV of all VCO samples showed high values of SV and ranged from 254.21 – 255.83 mg KOH/ g. The SV of all VCO samples aligned with the APCC (APCC, 2003) and Codex Alimentarius Commission (248-265 mg KOH/g oil) (Codex, 2003) standards for VCO and coconut oil. Mansor *et al.* (2012) reported that SV of VCO separated from coconut milk by enzymatic method using papain at 0.1% (w/w) of the milk had SV of 262.72 mg KOH/g oil. SV can be used to indicate the saponifiable matter, mainly fatty acids. High SV of VCO indicated the low impurity (Marina *et al.*, 2009).

All VCO samples had low values of IV with the range of 4.74-4.89 g I_2/100g oil. The IV aligned with the APCC (APCC, 2003) and Codex Alimentarius Commission (4.10 – 11.00 I_2/100g oil) (Codex, 2003) standards for VCO and coconut

oil. IV of VCO separated from coconut milk by enzymatic method using papain at 0.1% (w/w) had IV of 4.26 I_2/100g oil (Mansor *et al.*, 2012).

9.5.4 Effects of different proteases on emulsion destabilization of coconut milk

9.5.4.1 Emulsion stability

Emulsion stability of coconut milk treated with different proteases as monitored by creaming index (CI) is shown in Table 26. The CI provided indirect information about the extent of droplet aggregation in an emulsion. With higher CI, the greater particle aggregation occurs (Raghavendra and Raghavarao, 2010). Coconut milk treated with Alcalase showed the highest CI (p<0.05), followed by those treated with CPE, that incubated at 60°C for 90 min (control) and fresh coconut milk. CI in the coconut milk treated with Alcalase and CPE were 44.28±0.86% and 41.79±1.49%, respectively. The results indicated that aggregation of oil droplets took place to a high extent after being treated with CPE or Alcalase. Therefore, CPE or Alcalase played an important role in destabilisation of coconut milk emulsion. Proteins surrounding oil droplets were hydrolysed by protease, resulting in the liberation of oil or the formation cream (Sant'Anna et al., 2003). Raghavendra and Raghavarao (2010) reported that the coconut milk emulsion treated with and without aspartic protease had CI of 49.6 % and 27.7 %, respectively. For the control, creaming was also noticeable. Proteins acting as emulsifier might be denatured or coagulated during incubation for 90 min at 60°C. This led to instability of emulsion as indicated by creaming. For fresh coconut milk, the lowest CI was found. When coconut milk was allowed to stand at room temperature, oil droplet with low intensity could be floated and appeared as cream to some extent. Thus, CPE and Alcalaase could be used to destabilise emulsion of coconut milk for VCO production with the increased yield.

Table 26 Creaming index and particle size distribution of virgin coconut oil separated from coconut milk with the aid of crude protease extract from Pacific white shrimp hepatopancreas or Alcalase.

Treatments	Creaming index (%)	Particle size distribution	
		d_{32} (µm)	d_{43} (µm)
Fresh	33.33±2.28[d]	7.73±0.02[d]	19.26±0.01[d]
Control-90-min	37.81±0.86[c]	8.17±0.01[c]	25.95±0.01[c]
10-CPE-90-min	41.79±1.49[b]	12.21±0.01[b]	35.49±0.03[b]
10-Alc-90-min	44.28±0.86[a]	14.31±0.02[a]	45.49±0.02[a]

Means ± SD (n=3).

Different lowercase superscripts in the same column indicate significant differences ($p<$ 0.05). Fresh: fresh coconut milk; Control-90-min: control VCO (without protease and incubated at 60°C for 90 min); 10-CPE-90-min: VCO separated with CPE (10 unit/g protein) at 60°C for 90 min; 10-Alcalase-90-min: VCO separated with Alcalase (10 unit/g protein) at 60°C for 90 min.

9.5.4.2 Particle size distribution

Particle size distributions of coconut milk emulsion with various treatments are shown in Table 26. Coconut milk treated with Alcalase showed the largest oil droplets (d_{43} and d_{32}), followed by those treated by CPE. d_{32} in the coconut milk treated with Alcalase, CPE, the control and fresh coconut milk were 14.31±0.02, 12.21±0.01, 8.17±0.01 and 7.73±0.02 µm, respectively. Similar trend was also found for d_{43}, which were 45.49±0.02, 35.49±0.03, 25.95±0.01 and 19.26±0.01 µm for coconut milk treated with Alcalase, CPE, the control and fresh coconut milk, respectively. The d_{32} is related to the average surface area of droplet exposed to the continuous phase per unit volume of emulsion. The smaller d_{32} means the higher specific surface area, offering the increase in protein loads at interface of emulsions (Hebishy *et al.*, 2013; Intarasirisawat *et al.*, 2014). The d_{43} is the sum of the volume ratio of droplets in each size class multiplied by the mid-point diameter of size class. 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 (Intarasirisawat *et al.*, 2014). The highest d_{32} and d_{43} found in coconut milk treated

with Alcalase were in accordance with the highest creaming index of this sample. However, CPE was also shown to effectively destabilize the emulsion of coconut milk.

9.5.4.3 Microscopic structure

Microscopic structures of coconut milk emulsion subjected to treatment with different proteases were visualised by CLSM (Figure 28A). For the fresh coconut milk, the particulate clusters or clumps of oil droplets (red colour) in coconut milk emulsions were observed. It was noted that the smaller droplet size was obtained in fresh coconut milk (Figure 28Aa). However, the control sample incubated for 60°C for 90 min (without protease) (Figure 3Ab) had higher droplet size, compared with fresh coconut milk. After hydrolysis with CPE and Alcalase, the flocculation and coalescence took place in coconut milk emulsions, particularly those treated with Alcalase. CLSM image indicated that oil droplets were larger in sizes, especially that treated with Alcalase. Thus, the use of CPE and Alcalase was able to destabilise coconut milk emulsion.

Microscopic structures of coconut milk emulsion with different treatments were also visualized by phase contrast microscope (Figure 28B). For fresh coconut milk, the oil droplets were in uniform shape as well as size. Droplets were distributed evenly, indicating a stable emulsion (Figure 28Ba). When coconut milk subjected to the incubation at 60°C for 90 min, the oil droplets underwent coalescence and the larger droplets were formed (Figure 28Bb). After being treated with Alcalase or CPE at 60°C for 90 min, the larger oil droplets were observed (Figure 28Bc, 28Bd). The results clearly indicated that destabilization of emulsion was enhanced by protease as evidenced by the larger oil droplet. The similar result was reported for hydrolysis of coconut milk emulsion by aspartic protease (Raghavendra and Raghavarao, 2010). Thus, the use of CPE or Alcalase could induce the destabilization of coconut milk emulsion. This was associated with the release of free oil, which could be collected as VCO.

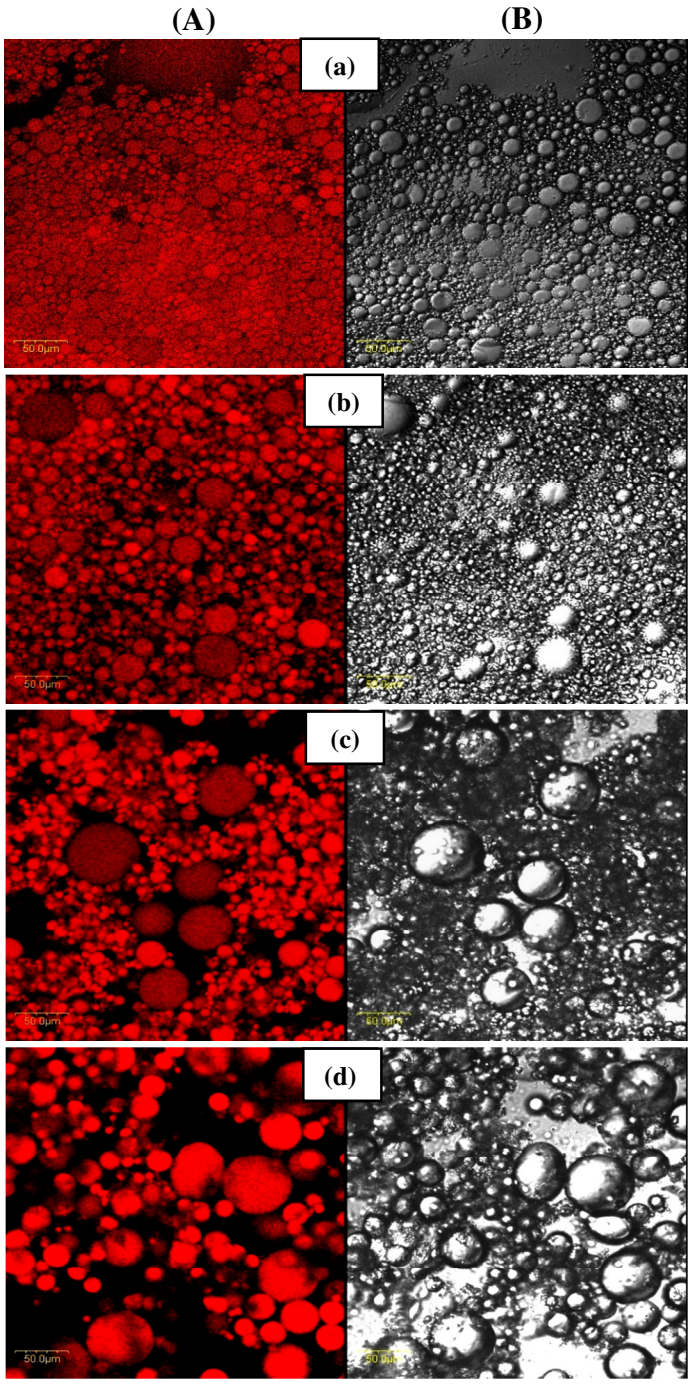

Figure 28 Confocal laser scanning micrographs (A) and phase contrast microscopy (B) of coconut milk emulsion with different treatments: (a) fresh coconut milk; (b) control (without protease and incubated at 60°C for 90 min); (c) VCO separated with CPE (10 unit/g protein) at 60°C for 90 min and (d) VCO separated with Alcalase (10 unit/g protein) at 60°C for 90 min. Magnification: 400×. Scale bar = 50 µm.

9.6 Conclusion

The separation of VCO using CPE or Alcalase (10 unit/g protein) at 60°C for 90 min increased the yield without negative effect on quality, particulary fatty acid composition. VCO had high contents of MCFAs, especailly lauric acid and myristic acid. Thus, the use of CPE or Alcalase at optimal temperature could increas the efficiency in VCO production and shorten the separation time.

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CHAPTER 10
CONCLUSION AND SUGGESTION

10.1 Conclusions

1. The protease from hepatopancreas of Pacific white shrimp was purified and classified as trypsin. Trypsin exhibited the highest hydrolytic activity toward BAPNA at 60 °C and pH 8.0.

2. The combined partitioning systems including TPP, ATPS and BE could be recovered and partially purified proteases from hepatopancrease of Pacific white shrimp.

3. Autolysis of hepatopancreas from Pacific white shrimp at 60 ^{o}C for 60 min was employed to increase the extraction yield without negative effect on fatty acid composition. Lipids obtained had high contents of DHA, EPA as well as astaxanthin.

4. Crude proteases extract (CPE) from hepatopancrease of Pacific white shrimp was able to hydrolyze the pretreated seabass skin effectively. The resulting protein hydrolysate exhibited various antioxidative activities, depending on the DH. CPE could be used as a replacer of commercial protease (Alcalase) for hydrolysate production.

5. Carotenoprotein extraction was achieved using CPE from Pacific white shrimp at 20 units/g shrimp shells with hydrolysis time of 120 min at 60 °C. Carotenoids extracted from carotenoprotein were rich in astaxanthin and showed strong antioxidant activity.

6. CPE of Pacific white shrimp at a level of 10 unit/g protein could be used as the extracting aid for lipid recovery from striped catfish mince. However the hydrolysis and oxidation of lipids occurred to some extent.

7. Virgin coconut oil (VCO) was extracted using CPE (10 unit/g protein) at room temperature (28-30 °C) for 6 h or at 60 °C for 90 min. The use of

CPE could shorthen the extraction time and the resulting VCO had the lower lipid oxidation and hydorlysis than that produced by the typical fermentation process. Thus, CPE could be used as promising source of protease for VCO production.

10.2 Suggestions

1. Trypsin of other fish and shellfish processing by-products should be recovered and studied.

2. Proteases from hepatopancreas of Pacific white shrimp recovered by the combined partitioning systems should be prepared in dried form in which the appropriate drying technology and stabilization should be investigated.

3. Other applications of proteases from hepatopancreas for enhanced hydrolysis of food proteins should be further studied.

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