Proteases from Pyloric Caeca of Brownstripe Red Snapper (*Lutjanus vitta*): Purification, Characterization and the Use for Production of Hydrolysate with Antioxidative Activity

Sutheera Khantaphant

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Author	Ms Sutheera Khantaphant
Major Program	Food Technology

Major Advisor:

……
(Prof. Dr. Soottawat Benjakul)

Examining Committee:

……...……...Chairperson
(Assoc. Prof. Dr. Aran H-Kittikun)

……..…….....
(Prof. Dr. Soottawat Benjakul)

……..…….....
(Assist. Prof. Dr. Manee Vittayanont)

……..
(Assist. Prof. Dr. Sappasith Klomklao)

The Graduate School, Prince of Songkla University, has approved this thesis as fulfillment of the requirements for the Doctor of Philosophy Degree in Food Technology.

……..……..…….
(Assoc. Prof. Dr. Krerkchai Thongnoo)
Dean of Graduate School

ชื่อวิทยานิพนธ์	โปรตีเอสจากไส้ติ่งปลากะพงข้างเหลือง (*Lutjanus vitta*): การทำบริสุทธิ์ การจำแนกลักษณะและการใช้เพื่อการผลิตโปรตีนไฮโดรไลเสตที่มีกิจกรรมการต้านออกซิเดชัน
ผู้เขียน	นางสาวสุธีรา ขันทพันธ์
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บทคัดย่อ

จากการศึกษาโปรตีเอสจากไส้ติ่งปลาของปลากะพงข้างเหลือง (*Lutjanus vitta*) ปลาตาหวานหนังหนา (*Priacanthus tayenus*) และปลาทรายแดง (*Nemipterus marginatus*) พบว่าโปรตีเอสจากไส้ติ่งปลาตาหวานหนังหนาและปลาทรายแดงมีกิจกรรมสูงสุดที่พีเอช 8.0 และอุณหภูมิ 60°ซ และพีเอช 8.5 และอุณหภูมิ 55°ซ ตามลำดับ โดยใช้เคซีน BAPNA และ TAME เป็นสับสเตรท ขณะที่โปรตีเอสจากไส้ติ่งปลากะพงข้างเหลืองมีกิจกรรมสูงสุดที่พีเอช 8.0 และอุณหภูมิ 60°ซ เมื่อใช้สับสเตรทดังกล่าว ยกเว้นเคซีนที่พบว่าโปรตีเอสจากไส้ติ่งมีกิจกรรมสูงสุดที่ 65°ซ การศึกษาผลของสารยับยั้งต่อกิจกรรมของโปรตีเอสจากไส้ติ่งของปลาทั้งสามสายพันธุ์ พบว่าโปรตีเอสมีลักษณะคล้ายทริปซิน โปรตีเอสจากไส้ติ่งของปลาทั้ง 3 สายพันธุ์ซึ่งแยกส่วนด้วยเกลือแอมโมเนียมซัลเฟตอิ่มตัวร้อยละ 40-60 ปรากฏเป็นแถบที่แสดงกิจกรรมการย่อยสลายโปรตีนเมื่อตรวจสอบโดยเทคนิค activity staining โดยแถบโปรตีนหลักมีน้ำหนักโมเลกุลเท่ากับ 24 22 และ 20 กิโลดาลตันตามลำดับ เมื่อใช้โปรตีเอสจากไส้ติ่งของปลาทั้งสามสายพันธุ์เพื่อผลิตเจลาตินไฮโดรไลเสตจากหนังปลาที่มีระดับการย่อยสลายร้อยละ 15 พบว่าเจลาตินไฮโดรไลเสตที่เตรียมด้วยโปรตีเอสจากไส้ติ่งปลากะพงข้างเหลืองแสดงกิจกรรมการต้านออกซิเดชันสูงสุด ($p < 0.05$)

โปรตีเอสจากไส้ติ่งปลากะพงข้างเหลืองผ่านการทำให้บริสุทธิ์ด้วยเทคนิคโครมาโทกราฟีต่าง ๆ จนได้แถบโปรตีนเดี่ยวบนเจล native-PAGE และ SDS-PAGE ซึ่งมีน้ำหนักโมเลกุล 23 กิโลดัลตัน เอนไซม์ที่ได้มีผลผลิตร้อยละ 4.9 และมีความบริสุทธิ์เพิ่มขึ้น 20 เท่า เอนไซม์บริสุทธิ์ที่ได้ถูกยับยั้งอย่างสมบูรณ์ด้วย SBTI และ TLCK เอนไซม์บริสุทธิ์มีกิจกรรมสูงสุดเมื่อใช้ BAPNA เป็นสับสเตรทที่พีเอช 8.5 และอุณหภูมิ 60°ซ และมีความคงตัวต่อพีเอชช่วง 7-10 และอุณหภูมิช่วง 25-55°ซ เอนไซม์บริสุทธิ์สามารถจำแนกเป็นทริปซิน ทริปซินมีความจำเพาะต่อการย่อยสลาย BAPNAและ TAME โดยมีค่า K_m เท่ากับ 0.507 และ 0.328 ไมโครโมลาร์ตามลำดับ และมีค่า k_{cat} เท่ากับ 4.71 และ 112 ต่อวินาที ตามลำดับ

เมื่อนำโปรตีเอสจากไส้ติ่งปลากะพงข้างเหลืองเพื่อเตรียมโปรตีนไฮโดรไลเสตจากกล้ามเนื้อปลากะพงข้างเหลือง โดยก่อนเริ่มกระบวนการย่อยสลาย กล้ามเนื้อปลากะพงข้างเหลืองผ่านการเตรียมเบื้องต้นโดยการกำจัดฟอสโฟลิปิดในส่วนเยื่อหุ้มเซลล์และสารเร่งปฏิกิริยาออกซิเดชัน ซึ่งพบว่าโปรตีนไฮโดรไลเสตจากกล้ามเนื้อปลากะพงข้างเหลืองที่ผ่านการเตรียมเบื้องต้นแสดงกิจกรรมการต้านออกซิเดชันสูงกว่าโปรตีนไฮโดรไลเสตจากกล้ามเนื้อปลากะพงข้างเหลืองและโปรตีนไฮโดรไลเสตจากโปรตีนไอโซเลทของกล้ามเนื้อปลากะพงข้างเหลืองที่ผ่านการเตรียมเบื้องต้นที่ทุกระดับการย่อยสลายที่ศึกษา ($p < 0.05$) นอกจากนี้โปรตีนไฮโดรไลเสตจากกล้ามเนื้อปลากะพงข้างเหลืองที่ผ่านการเตรียมเบื้องต้นที่ระดับการย่อยสลายร้อยละ 40 ยังแสดงสมบัติการเป็นสารต้านออกซิเดชันในระบบเลซิทิน-ไลโพโซม กล้ามเนื้อปลากะพงข้างเหลืองที่ผ่านการเตรียมเบื้องต้นถูกย่อยสลายด้วยวิธีการย่อยสลาย 2 ขั้นตอน โดยเริ่มการย่อยสลายในขั้นแรกด้วยเอนไซม์อัลคาเลสหรือฟลาโวไซม์จนได้โปรตีนไฮโดรไลเสตที่มีระดับการย่อยสลายร้อยละ 40 และตามด้วยการย่อยสลายในขั้นตอนที่ 2 ด้วยโปรตีเอสจากไส้ติ่งปลากะพงข้างเหลือง เป็นเวลา 2 และ 1 ชั่วโมง เพื่อให้ได้โปรตีนไฮโดรไลเสตที่ผ่านการย่อยสลายด้วยเอนไซม์อัลคาเลสและโปรตีเอสจากไส้ติ่ง (HAP) และโปรตีนไฮโดรไลเสตที่ผ่านการย่อยสลายด้วยเอนไซม์ฟลาโวไซม์และโปรตีเอสจากไส้ติ่ง (HFP) ตามลำดับ HAP แสดงกิจกรรมการกำจัดอนุมูลอิสระ DPPH และ ABTS และกิจกรรมการรีดิวซ์เฟอริก (FRAP) ที่สูงกว่า ในขณะที่ HFP แสดงกิจกรรมการจับโลหะ (FE^{2+}) และกิจกรรมการยับยั้งเอนไซม์ ACE ที่สูงกว่า ($p < 0.05$) ทั้ง HAP และ HFP แสดงสมบัติการเป็นสารต้านออกซิเดชันแปรผันโดยตรงกับความเข้มข้นในระบบเลซิทิน-ไลโพโซมและระบบบีตา-แคโรทีน กรดลิโนเลอิค ตลอดจนต้านออกซิเดชันในระบบเนื้อปลาบด การย่อยสลายในระบบจำลองทางเดินอาหารทำให้กิจกรรมการต้านออกซิเดชันของทั้ง HAP และ HFP เพิ่มขึ้น ยกเว้นกิจกรรมการกำจัดอนุมูลอิสระ DPPH ซึ่งชี้กิจกรรมการต้านออกซิเดชันที่เพิ่มขึ้นภายหลังการย่อยในระบบทางเดินอาหาร

การศึกษาการแยกเปปไทด์ที่มีกิจกรรมต้านออกซิเดชันใน HAP และ HFP ด้วยเทคนิคโครมาโทกราฟีต่าง ๆ ซึ่งประกอบด้วยคอลัมน์ Sephadex G-25 และ Phenogel™ พบว่าเปปไทด์ที่แสดงกิจกรรมการกำจัดอนุมูลอิสระ ABTS มีลำดับกรดอะมิโนคือ Asn-Arg-Lys-Arg, Asp-Ala-Gly-Leu-Phe-Lys, Met-Glu-Glu-Asp-Leu-Glu-Glu-Arg และ Met-Ser-Leu-Trp-Gln-Ser-Leu-Met-Asn-Asp-Lys ขณะที่เปปไทด์ที่แสดงกิจกรรมการจับโลหะ (Fe^{2+}) มีลำดับกรดอะมิโนคือ Cys-Gly-Asp-Ser-Val-Lys, Met-Cys-Cys-Cys-Arg, His-Arg-Arg-Arg, Asn-Phe-Cys-Ser-Arg, Trp-Trp-Arg-Lys และ Phe-Cys-Gly-Val-Ala-Thr-Lys

HAP และ HFP ประกอบด้วยโปรตีนในปริมาณสูง (ร้อยละ 87.36 และ 86.55 ตามลำดับ) กรดอะมิโนหลักที่เป็นองค์ประกอบของ HAP และ HFP คือกรดกลูตามิก/กลูตามีน นอกจากนี้ยังประกอบด้วยกรดอะมิโนแอสปาร์ติก/แอสพาราจีน ไลซีน อะลานีน และลูซีน ในปริมาณสูง HAP และ HFP มีความสามารถในการละลายมากกว่าร้อยละ 98 ในช่วงพีเอช 2-12 HAP และ HFP สามารถแสดงสมบัติระหว่างเฟสโดยขึ้นกับความเข้มข้น อย่างไรก็ตามสมบัติการเป็นอิมัลชันและสมบัติการเกิดฟองของ HFP ดีกว่า HAP ($p < 0.05$) โดยทั่วไปกิจกรรมการต้านออกซิเดชันของ HAP และ HFP มีความคงตัวในช่วงอุณหภูมิ 25-100^{o}ซ และช่วงพีเอช 2-12 แต่พบว่ากิจกรรมการกำจัดอนุมูลอิสระ ABTS มีค่าลดลงเล็กน้อยเมื่อพีเอชอยู่ในช่วงด่างสูง ($p < 0.05$) จากการเก็บรักษา HAP และ HFP ที่อุณหภูมิ 4^{o}ซ เป็นเวลา 12 สัปดาห์ พบว่ากิจกรรมการต้านออกซิเดชันมีค่าลดลงเล็กน้อยเมื่อระยะเวลาการเก็บรักษานานขึ้นและโปรตีนไฮโดรไลเสตมีสีเหลืองเพิ่มขึ้น ($p < 0.05$) เมื่อเติม HAP และ HFP ที่เตรียมใหม่หรือที่เก็บรักษานาน 8 เดือนในน้ำนมถั่วเหลืองที่ระดับร้อยละ 0.3 หรือ 0.4 (น้ำหนักต่อปริมาตร) ตามลำดับ พบว่าน้ำนมถั่วเหลืองเสริมโปรตีนไฮโดรไลเสตแสดงกิจกรรมการต้านออกซิเดชันที่เพิ่มขึ้นโดยไม่พบความแตกต่างด้านคะแนนความชอบรวมเมื่อเทียบกับน้ำนมถั่วเหลืองชุดควบคุม

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ABSTRACT

Proteases from pyloric caeca of brownstripe red snapper (*Lutjanus vitta*), bigeye snapper (*Priacanthus tayenus)* and threadfin bream (*Nemipterus marginatus*) were studied. Those from bigeye snapper and threadfin bream exhibited the highest hydrolytic activities toward casein, α-*N*-benzoyl-DL-arginine-*p*-nitroanilide (BAPNA) and α-*N*-*ρ*-toluene-sulfonyl-L-arginine methyl ester (TAME) at pH 8.0 and 60^{o}C and pH 8.5 and 55^{o}C, respectively, while that from brownstripe red snapper showed the optimal pH and temperature of 8.0 and 60^{o}C with all substrates used except the optimal temperature of 65^{o}C for casein. The protease inhibition study suggested that those proteases were trypsin-like. Based on activity staining, pyloric caeca proteases (PCPs) obtained after precipitation with 40-60% ammonium sulfate showed the major activity bands with molecular weight (MW) of 24, 22 and 20 kDa, respectively. PCPs were used for fish skin gelatin hydrolysis with the degrees of hydrolysis (DH) of 15%. Among all gelatin hydrolysates, that prepared using PCP of brownstripe red snapper exhibited the highest antioxidative activities ($p < 0.05$).

PCP from brownstripe red snapper was subsequently subjected to soybean trypsin inhibitor (SBTI) affinity column and DEAE column chromatography. Purified protease showed a single band on SDS-PAGE and native-PAGE with the yield of 4.9 % and 20-fold in purity with MW of 23 kDa. Purified protease was strongly inhibited by SBTI and *N*-*ρ*-tosyl-L-lysine chloromethylketone (TLCK). The maximal activity toward BAPNA was observed at pH 8.5 and 60°C and was stable within temperature range of 25-55°C and pH range of 7.0-10.0. The purified protease was therefore classified as trypsin. Trypsin had K_m for hydrolysis of BAPNA and TAME of 0.507

and 0.328 mM, respectively and k_{cat} of 4.71 and 112 s^{-1} were obtained for BAPNA and TAME hydrolysis, respectively.

PCP from brownstripe red snapper was used to prepare the protein hydrolysate from the muscle of the same species. Prior to hydrolysis, the pretreatment including phospholipid membrane separation with subsequent washing was performed. The hydrolysate from pretreated mince showed the higher antioxidative activities, compared to those of untreated mince and protein isolate from pretreated mince at all DH tested ($p < 0.05$). Hydrolysate from pretreated mince (40% DH) could retard the oxidation in liposome oxidation system, compared with the control ($p < 0.05$). Thereafter, protein hydrolysate from the pretreated muscle was prepared by two-step hydrolysis process using Alcalase or Flavourzyme for the first step with 40% DH, followed by hydrolysis with PCP as the second step for 2 h (HAP) and 1 h (HFP), respectively. HAP exhibited the higher DPPH and ABTS radical scavenging activity and ferric reducing antioxidant power, while HFP showed the higher ferrous chelating activity and ACE inhibitory activity ($p < 0.05$). Both HAP and HFP were able to retard lipid oxidations in lecithin-liposome and β-carotene-linoleic acid model systems in dose-dependent manner and also retarded the oxidation in comminuted fish systems. After being subjected to gastrointestinal tract model system, antioxidative activities, except DPPH radical scavenging activity, of both HAP and HFP increased, indicating the enhancement of antioxidative activities after ingestion.

HAP and HFP were isolated for their antioxidative peptides using a series of chromatographies, including Sephadex G-25 column and Phenogel^{TM} column. Peptides showing ABTS radical scavenging activities in HAP were identified as Asn-Arg-Lys-Arg, Asp-Ala-Gly-Leu-Phe-Lys, Met-Glu-Glu-Asp-Leu-Glu-Glu-Arg and Met-Ser-Leu-Trp-Gln-Ser-Leu-Met-Asn-Asp-Lys. Ferrous chelating peptides derived from HFP were Cys-Gly-Asp-Ser-Val-Lys, Met-Cys-Cys-Cys-Arg, His-Arg-Arg-Arg, Asn-Phe-Cys-Ser-Arg, Trp-Trp-Arg-Lys and Phe-Cys-Gly-Val-Ala-Thr-Lys.

HAP and HFP contained high protein content (87.36 and 86.55%, respectively). Glutamic acid/glutamine were the major amino acids in both HAP and HFP. Both hydrolysates were also rich in aspartic acid/asparagine, lysine, alanine and

leucine. HAP and HFP had the high solubility greater than 98% over a wide pH range (2-12). Both HAP and HFP exhibited interfacial properties in dose-dependent manner, however HFP had the higher emulsifying and foaming properties than did HAP (p <0.05). Antioxidative activities of both hydrolysates were generally stable over a wide temperature range (25-100°C). High stability over a wide pH range (2-12) was observed for both HAP and HFP, but ABTS radical scavenging activity slightly decreased in very alkaline pH ranges ($p < 0.05$). Antioxidative activities of HAP and HFP slightly decreased during storage at 4°C for 12 weeks with coincidental increase in yellowness (b^* value) (p <0.05). Freshly prepared or 8-week stored HAP and HFP at the level of 0.3 and 0.4%, respectively could enhance the antioxidative activities of fortified soybean milk. No differences in overall likeness were obtained, compared with the original soybean milk.

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Sutheera Khantaphant

CONTENTS

CONTENTS (Continued)

Chapter **Page**

CONTENTS (Continued)

CONTENTS (Continued)

Chapter **Page**

CONTENTS (Continued)

Chapter **Page**

CONTENTS (Continued)

Chapter **Page**

CONTENTS (Continued)

Chapter **Page**

LIST OF TABLES

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CHAPTER 1

INTRODUCTION AND REVIEW OF LITERATURE

1.1 Introduction

Lipid oxidation is one of the major deteriorations in many types of natural and processed food, leading to the changes in food quality and nutritional value and also produces potentially toxic reaction products (Flaczyk *et al*., 2006). Furthermore, DNA, cell membranes, and proteins are target sites of the lipid oxidation processes, and consequently induce different kinds of serious human diseases, such as cardiovascular disease, cancer, and neurological disorders as well as the aging process (Jittrepotch *et al*., 2006). To prevent foods from deterioration and to provide protection against serious diseases, it is important to inhibit the oxidation of lipids and formation of free radicals occurring in the living body and foodstuffs. Thus, antioxidants are required. The commonly used antioxidants are mostly chemically synthesized antioxidants such as butylated hydroxyanisole (BHA), butylated hydroxytoluene (BHT) and tert-butylhydroquinone (TBHQ). However, these antioxidants have been suspected for their toxicity, carcinogenic and DNA damage induction. As a consequence, their use in foodstuffs is restricted or prohibited in some countries (Sakanaka *et al*., 2004; Je *et al*., 2005). Therefore, there is a growing interest on natural additives as potential alternative antioxidants, especially antioxidative peptides derived from proteinaceous sources such as plant and animal proteins.

Hypertension, the term of high blood pressure has been considered the most common serious chronic health problem (Jung *et al*., 2006a, Bougatef *et al*., 2008b; Raghavan and Kristinsson, 2009). Since angiotensin I-converting enzyme (ACE) (EC 3.4.15.1) is physiologically important in raising blood pressure, inhibition of ACE activity can lead to an overall antihypertensive effect. The synthetic ACE inhibitors are now widely used as pro-drugs but these synthetic ACE inhibitors can cause many significant undesirable side effects, such as coughing, allergic reactions, taste disturbances, and skin rashes (Bougatef *et al*., 2008b; Raghavan and Kristinsson,

2009). Therefore, the natural safe compounds are desirable for prevention of free radical from oxidation and hypertension instead of the synthetic counterpart.

Hydrolysis processes have been widely used to convert under-utilized proteins into more marketable and acceptable forms (Klompong *et al*., 2009). Hydrolysis is commonly achieved with the aid of enzymes, which produces better functional and nutritional properties of food proteins (Kristinsson and Rasco, 2000b; Limam *et al*., 2008). However, hydrolysates from muscle protein sources can have several pro-xidants such as heme and unstable oxidized lipid substrates. (Chaijan *et al*., 2005; Raghavan *et al*., 2008). These compounds may not only decrease the stability of protein hydrolysates but may also affect their bioactivity (Raghavan and Kristinsson, 2009). The removal of these pro-oxidants from protein source before hydrolysis therefore provide the hydrolysates with high quality and stability. Enzymatic hydrolysed proteins from various fish have been reported to possess antioxidative activities and ACE inhibitory activity.

In Thailand, brownstripe red snapper (*Lutjanus vitta*) is one of the main raw materials for surimi production (Jongjareonrak *et al*., 2006). Apart from being processed into surimi, its flesh and gelatin from its skin can be used as the raw material for production of protein hydrolysate with bioactivities. Moreover, fish viscera generated during fish processing can be used as the rich sources of various enzymes, especially trypsin (Klomklao *et al*., 2007b). Therefore, proteases, mainly trypsin from pyloric caeca of brownstripe red snapper could be used as the potential aid for the preparation of protein hydrolysate with antioxidative activity as well as ACE inhibitory activity. Furthermore, using proteases from fish viscera has an advantage over commercial proteases since enzyme costs can be greatly reduced (Shahidi *et al*., 1995). Production of fish protein hydrolysates with antioxidative and/or ACE inhibitory activities can pave the way for full utilization of this species and produce the new value-added product of health benefit with high market value. Furthermore, the production of protein hydrolysate with bioactive activities can be the promising means to maximize the utilization of this species and also minimize the pollution caused by waste disposal. Additionally, the information gained can be of benefit for fish processing industry and functional food industry of Thailand.

1.2 Review of literature

1.2.1 Fish proteases

During fish processing, large amounts of waste including viscera are generated. Fish viscera are non-edible parts produced in large quantities and become a waste or pollution (Kishimura *et al*., 2008). These by-products are recognized as a potential source of digestive enzymes, especially proteases (Klomklao *et al*., 2007b) that may be of industrial applications (Simpson, 2000), such as scale removal from fish skin or cleaning fish instead of using mechanical procedures and as the processing aids in fishmeal production. According to the International Union of Applied Biochemists classification, proteases from fish and aquatic invertebrates may be classified into four major groups (Simpson, 2000).

1.2.1.1 Classification of fish proteases

1.2.1.1.1 Aspartiyl proteases

Aspartly or acid proteases have been described as a group of endopeptidase characterized by high activity and stability at acid pH. Their catalytic sites are composed of the carboxyl groups of two aspartic acid residues (Simpson, 2000). Based on the EC system, all the 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). Among these aspartyl proteases, pepsin is the major enzyme for this group. Pepsin (EC 3.4.23.1) is secreted as pepsinogen from chief cells of oxyntic glands located in the stomach wall epithelium. When pepsinogen is in acidic environment, it rapidly converts to pepsin (Kageyama, 2002). During this activation reaction, both prosegment (activation segment) and the active enzyme undergo the conformational changes, and the proteolytic cleavage of the prosegment can occur in one or more steps by either an intra or intermolecular reaction (Nalinanon *et al*., 2010). Pepsin prefers specifically the aromatic amino acids phenylalanine, tyrosine and tryptophan. Fish pepsin have been isolated from various

fish species such as Atlantic cod (Gildberg *et al*., 1990), Monterey sardine (Castillo-Yañez *et al*., 2004), sea bream (Zhou *et al*., 2007), pectoral rattail (Klomklao *et al*., 2007e), smooth hound (Bougatef *et al*., 2008a) and European eel (Wu *et al*., 2009).

1.2.1.1.2 Serine proteases

The serine proteases are a group of endopeptidase with a serine residue together with an imidazole group and an aspartyl carboxyl group in their catalytic sites (Simpson, 2000). The proteases in serine subclass have the same first three digits on the EC system as EC 3.4.21. Serine proteases exhibit high activity under alkaline rather than neutral pH and sensitivity to serine protease inhibitors (Simpson, 2000). The common serine proteinases have been recovered from fish viscera are trypsin (EC 3.4.21.4), chymotrypsin (EC 3.4.21.1) and elastase (EC 3.4.21.11) (Simpson, 2000). Trypsin has a very narrow specificity for the peptide bonds on the carboxyl side of arginine and lysine, while chymotrypsin has a much broader specificity for amino acids with bulky side chains and nonpolar amino acids such as tyrosine, phenylalanine, tryptophan and leucine. Elastase exhibits preferential specificity for alanine, valine and glycine (Simpson, 2000). Trypsin has been isolated and characterized from the viscera of various fish species, including Monterey sardine (Castillo-Yanez *et al*., 2005), chinook salmon (Kurtovic *et al*., 2006), bigeye snapper (Hau and Benjakul, 2006), bluefish (Klomklao *et al*., 2007d) and walleye pollock (Kishimura *et al.,* 2008).

1.2.1.1.3 Cysteine proteases

Cysteine or thiol proteases are a group of endo peptidase that have cysteine and histidine residues as the essential groups in their catalytic sites (Simpson, 2000). The first three digits on the EC system of cysteine proteases are EC 3.4.22. An example of a thiol protease from the digestive glands of marine animals is cathepsin B (EC 3.4.22.1) (Simpson, 2000). 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).

1.2.1.1.4 Metalloproteases

Metalloproteases are hydrolytic enzymes whose activity depends on the presence of bound divalent cations at their active sites (Simpson, 2000). The metalloproteases have a common first three digits as EC 3.4.24 (Simpson, 2000). Generally, metalloproteases are inhibited by chelating agent such as 1, 10-phenanthroline and EDTA. Collagenolytic metalloprotease (gelatinase) was isolated from hepatopancreas of the marine crab (Sivakumar *et al*., 1999).

Digestive proteases are hydrolytic in their action and catalyze the cleavage of peptide bonds specifically (Simpson, 2000). The most important proteolytic enzymes from fish viscera are the aspartic protease, e.g. pepsin, and serine proteases, e.g. trypsin, chymotrypsin and elastase (Aspmo *et al*., 2005). Aspartic proteases, normally obtained from fish stomachs, display high activity between pH 2 and 4, while serine proteases are most active between pH 8 and 10 (Simpson, 2000). Aspartic proteases, especially pepsin, were mainly used for hydrolysis purposes, such as the preparation of protein isolate and the extraction of collagen and gelatin (Nalinanon *et al*., 2010). The use of serine proteases, especially trypsin, has increased remarkably due to the stability and activity under harsh conditions, such as temperatures of 50-60°C, high pH values as well as in the presence of surfactants or oxidizing against surfactants (Klomklao *et al*., 2007b).

1.2.1.2 Fish trypsins

Trypsin (EC 3.4.21.4) is one of the main digestive proteases in the group of serine proteases (Simpson, 2000). Trypsin has a catalytic triad of three essential amino acid residues includes serine (Ser), histidine (His) and aspartate (Asp) within the S1 binding pocket. The high specificity of trypsin for lysine and arginine results from the negative charge of Asp at the S1 binding pocket of trypsin matching the positive charge of the P1 side chain of the substrate (Kishimura *et al*., 2010). In the pancreas, 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 (Kishimura *et al*., 2010). Fish trypsin has low thermal stability but shows high activity at low temperature, especially, for cold

water species. Furthermore, it is unstable at acid pH, and contains a lower content of basic amino acid residues in the polypeptide chain in comparison with mammalian trypsins (Simpson, 2000). Trypsin consists of single peptide chain with molecular weight (MW) typically 24 kDa (Torrissen and Male, 2000). The 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 enzyme, cleaving peptide bonds at the carboxyl terminus of lysine and arginine residues exclusively (Torrissen and Male, 2000).

Trypsins have been isolated and characterized from the pyloric caeca, pancreatic tissue, or intestines of several marine animals. Kishimura and Hayashi (2002) reported that MW of purified trypsin from pyloric caeca of the starfish was 28 kDa. Optimal pH and temperature of trypsin were pH 8.0 and 55°C, respectively. Trypsin from pyloric caeca of Monterey sardine was purified and the MW was 25 kDa. The optimal pH and temperature was pH 8.0 and 50°C, respectively. (Castillo-Yañez *et al.*, 2005). Hau and Benjakul (2006) purified a 23.8 kDa trypsin from the pyloric caeca of bigeye snapper and the maximal activity was found at 55°C and pH range of 8-10. Trypsin from pyloric caeca of chinook salmon was isolated (Kurtovic *et al.*, 2006). The enzyme was active over a broad pH range (7.5-10.0) with the optimal temperature at 60°C and the MW was 28 kDa. A 26 kDa trypsin was isolated from pyloric caeca of New Zealand hoki with optimal temperature and pH of 60°C and 9.0, respectively (Shi *et al*., 2007). Trypsins from the pyloric caeca of jacopever and elkhorn sculpin showed the optimal temperature at 60°C and 50°C, respectively. Both trypsins showed the same optimal pH at 8.0 and had the same MW of 24 kDa (Kishimura *et al.*, 2007). Klomklao *et al*. (2007d) purified trypsin from pyloric caeca of bluefish and the MW of 28 kDa was found. Trypsin from pyloric caeca of walleye pollock was purified and the MW of enzyme was estimated to be 24 kDa. At pH 8.0 and 50°C, walleye pollock exhibited the highest hydrolysis activity (Kishimura *et al*., 2008). Trypsin A and B purified from the pyloric caeca of mandarin fish with MW of 21 and 21.5 kDa, respectively, exhibited the maximal activity at 35°C and 40°C, respectively, at pH 8.5 (Lu *et al*., 2008). Kishimura *et al*. (2010) purified trypsin from pyloric caeca of threadfin hakeling. The MW of the trypsin was 24 kDa and showed

the maximal activities at pH 8.0 and 50°C. Trypsins from pyloric caeca of Pacific cod and saffron cod were purified. Both enzymes showed the MW of 24 kDa and the optimal pH and temperature were 8.0 and 50°C, respectively (Fuchise *et al*., 2009). Trypsin from the intestine of hybrid tilapia was also purified and the MW of 22 kDa was obtained with the optimal pH and temperature of 9.0 and 60°C, respectively (Wang *et al*., 2010). Additionally, trypsin from the intestine of smooth hound was purified (Bougatef *et al*., 2010a). Trypsin had the estimated MW of 24 kDa and the optimal pH and temperature of 8.5 and 50°C, respectively. The optimal conditions and substrates of fish trypsins are summarized in Table 1.

1.2.2 Lipid oxidation and generation of free radicals

Oxidation is the transfer of electrons from one atom to another. It is an essential part of normal metabolism for the survival of cells. However, serious problems arise when electrons are transferred not in pairs, but escape the process as unpaired single electrons. A side effect of this dependence is the production of unpaired electrons called free radicals (Block, 1999). The most abundant radical in biological system is oxygen species (Reilly *et al*., 1991). The harmful effect of free radicals occurs in bological systems when there is an overproduction of reactive oxygen species (ROS) on one side and a deficiency of enzymatic and non-enzymatic antioxidants on the other (Valko *et al.*, 2007). Environmental factors such as pollution, radiation, cigarette smoke, herbicides, pesticides and eventhough physical stress can also generate free radicals (Kaur and Kapoor, 2001). The human body possesses many defence mechanisms against free radicals including antioxidant enzymes (superoxide dismutase, catalase and peroxidase) and non-enzymatic compounds (glutathione, urate and coenzyme Q) (Halliwell, 2006). Nevertheless an excess of these harmful molecules can be out of control and cause destructive and lethal cellular effects by oxidizing membrane lipids, cellular proteins, DNA and enzymes, thus shutting down cellular respiration (Pihlanto, 2006). Additionally, the susceptibility of lipid to oxidation is one of the major deterioration in many types of natural and processed food (Jittrepotch *et al*., 2006) and is of great concern for the

Table 1. Optimal condition and substrates of various fish trypsins

Identified species	Optimum pH	Optimum temperature (ºC)	Substrates	References
Starfish (*Asterina Pectinifera*)	8	55	TAME[a]	Kishimura and Hayashi (2002)
Japanese anchovy (*Engraulis japonica*)	8	60	TAME	Kishimura *et al*. (2005)
Monterey sardine (*Sardinops sagax caerulea*)	8	50	BAPNA[b]	Castillo-Yañez *et al.* (2005)
Bigeye snapper (*Pricanthus macracanthus*)	8-11	55	BAPNA	Hau and Benjakul, 2006
Chinook salmon (*Oncorhynchus tshawytscha*)	8	60	BAPNA	Kurtovic *et al*. (2006)
New Zealand hoki (*Macruronus Novaezealandlae*)	9	60	BAPNA	Shi *et al*. (2007)
Bluefish (*Pomatomus saltatrix*)	9.5	55	BAPNA	Klomklao *et al*. (2007d)
Skipjack tuna (*Katsuwonus pelamis*)	8.5	60	TAME	Klomklao *et al*. (2007b)
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)
Threadfin hakeling (*Laemonema longipes*)	8	50	TAME	Kishimura *et al*. (2010)
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*. (2010)
Smooth hound (*Mustelus mustelus*)	8.5	50	TAME	Bougatef *et al*. (2010a)

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

food industry (Sakanaka *et al.*, 2004) because lipid oxidation leads to changes in the quality of food such as taste, texture, appearance, and nutritional value and many undesirable properties such as off-flavors, odors, dark colors and potentially toxic reaction products.

Lipid oxidation is a complex process, whereby unsaturated fatty acids react with molecular oxygen via a free radical chain mechanism, and form fatty acyl hydroperoxides as primary products in the oxidation processes. Lipid oxidation classically proceeds in three phases: (Cheeseman, 1993).

1) Initiation

Lipid oxidation must start with the abstraction of a hydrogen atom from the target fatty acid to form a lipid radical ($L^{\bullet}$). The initiating radical could be any radical species sufficiently react with polyunsaturated fatty acids (PUFAs). The initiators for lipid radical formation are trace metals, irradiation, light or heat (Cheeseman, 1993). Hydroxyl radical ($OH^{\bullet}$), peroxyl radicals ($ROO^{\bullet}$) and alkoxyl radicals ($RO^{\bullet}$) are capable of initiating lipid oxidation. The primary product of lipid oxidation, lipid hydroperoxide (LOOH), can undergo cleavage to form alkoxyl radicals ($LO^{\bullet}$) or peroxyl radicals ($LOO^{\bullet}$).

2) Propagation

The initiation of a new chain by a resulting free radical is the propagation stage of lipid oxidation. $L^{\bullet}$ and $LOO^{\bullet}$ generated can initiate a chain reaction with other molecules, resulting in the formation of lipid free radicals and lipid hydroperoxides (LOOH) which are not very stable (Jadhav *et al*., 1996). The presence of metal ions, especially Fe^{2+}, leads to the breakdown of lipid hydroperoxides and generates free radical species capable of initiating new chains of lipid oxidation (Cheeseman, 1993).

3) Termination

The radical-radical reactions also occur when the conditions are favorable and these have the effect of terminating the chains of lipid oxidation since

non-radical compounds are produced. These reactions require the individual reactants to be present at relatively high concentrations to make their interaction feasible. It should also be noted that the reaction of two lipid peroxyl radicals may yield singlet oxygen, a reactive species (Cheeseman, 1993).

1.2.3 Antioxidants

Antioxidant is, by food scientists, the term implicitly restricted to chain-breaking inhibitors of lipid peroxidation. A broader definition of an antioxidant is any substance that when present at low concentrations compared with those of an oxidizable substrate, significantly delays or prevents oxidation of that substrate (Halliwell, 1995). Antioxidants neutralize free radicals by donating one of their own electrons, ending the electron-stealing reaction. The antioxidants do not themselves become free radicals by donating electrons because they are stable in both forms. They may well be defined as the substances that are capable of scavenging or quenching or stabilizing free radicals. (Kaur and Kapoor, 2001).

1.2.3.1 Classification of antioxidants

1.2.3.1.1 Primary antioxidant

Primary antioxidant (type 1 or chain-breaking antioxidants) is free radical acceptor that delays or inhibits the initiation step or interrupts the propagation step of oxidation (Reische *et al*., 1998). It controls the radical chain reaction by donating hydrogen atom or electron to free radicals ($R^{\bullet}$) and produces the derivatives (RH) and antioxidant radicals ($A^{\bullet}$) that are more stable and less readily available to react with lipids or oxygen molecule so that it can reduce rate of propagation (Jittrepotch *et al*., 2006). Primary antioxidant is effective at very low concentration (Rajalakshmi and Narasimhan, 1996). Most of primary antioxidants are synthetic compounds, such as butylated hydroxyanisole (BHA), butylated hydroxytoluene (BHT), propyl gallate (PG) and tertiary butylhydroquinone (TBHQ). Natural primary antioxidants are tocopherols and carotenoids (Reische *et al*., 1998).

1.2.3.1.2 Secondary antioxidant

Secondary antioxidant (type 2 or preventive antioxidant) is compound that slows the rate of oxidation by several different actions, but they do not convert free radicals to more stable compounds (Reische *et al*., 1998). Those antioxidants can chelate pro-oxidant metals, replenish hydrogen atom to primary antioxidants, decompose hydroperoxides into non-radical species, deactivate singlet oxygen, absorb UV radiation and act as oxygen scavengers. This type of antioxidant is also usually called as synergists because they can promote the activity of primary antioxidants. Normally, the secondary antioxidants only show antioxidative activity when a second minor component is present. For example, sequestering agents such as citric acid are effective only in the presence of metal ions, and reducing agents such as ascorbic acid are effective in the presence of tocopherols or other primary antioxidants (Gordon, 2001).

Since artificial antioxidants possess potential risks *in vivo*, their use in foodstuffs is restricted or prohibited in some countries (Pihlanto, 2006). The considerable interest has arisen in finding alternative sources of antioxidants for use in food systems. Natural antioxidants therefore were taken into consideration.

1.2.3.2 Sources of natural antioxidants

1.2.3.2.1 Plant extracts

Potential antioxidants in plants can be found in some pigments (fucoxanthin, astaxanthin, carotenoid) and polyphenols (phenolic acid, flavonoid, tannins) (Heo *et al.*, 2005). Phenolic compounds are the major contributors to the antioxidative activities of plants Zainol *et al.* (2003) considered that phenolic compounds from different parts of plant possess the different antioxidative activities. The antioxidant properties of phenolics are mainly because of their redox properties, which allow them to act as reducing agents, hydrogen donators and singlet oxygen quenchers (Rice-Evans *et al.*, 1997). These phenolic compounds, during lipid oxidation, act in various ways, such as binding metal ions, scavenging radicals or decomposition of peroxides (Moure *et al.*, 2001). Jayaprakasha *et al.* (2001) reported the antioxidative activity of grape seed extracts by preventing the peroxidation of

linoleic acid in model system. Abdille *et al.* (2005) reported that phenolic compounds with ortho- and para-dihydroxylation or with a hydroxy and a methoxy group are more effective than simple phenolics. Many classes of phenolic compounds in barley showed strong antioxidant activities (Liu and Yao, 2007). Antioxidative activity in plants generally increased with increasing content of phenolic compound (Abdille *et al.*, 2005).

1.2.3.2.2 Maillard reaction products and caramelization products

During processing and storage, non-enzymatic browning reactions can occur in food. Products of browning reactions, both Maillard reaction and caramelization, have been shown to possess the antioxidant activity. Maillard reaction products (MRPs) possess the radical scavenging activity (Marales and Jimenez-Perez, 2001) as well as chelating properties (Yoshimura *et al.*, 1997). Benjakul *et al.* (2005b) prepared MRPs from porcine plasma protein and reducing sugars (glucose, fructose and galactose) and found that MRPs derived from galactose possessed greater antioxidative activities than those prepared from fructose and glucose. Caramelization products (CPs) also exhibit the antioxidative activity. Benjakul *et al.* (2005a) determined the antioxidative activity from different sugars (glucose, fructose, ribose and xylose) prepared by heating at 100°C. CPs from fructose exhibited the highest antioxidative activity with radical scavenging activity and reducing power. Generally, MRPs with brown color showed the increased antioxidative activity with increasing concentration (Benjakul *et al.*, 2005b; Lertittikul *et al.*, 2007).

1.2.3.2.3 Peptides and protein hydrolysates

Protein hydrolysate, peptides and amino acids have been found to possess antioxidative activity (Amarowicz and Shahidi, 1997). Numerous peptides derived from hydrolyzed food proteins have been shown to have noteworthy antioxidative activities against the peroxidation of lipids or fatty acids. Whey and soy protein hydrolysates had the inhibitory effect against lipid oxidation in liposome system (Peña-Ramos and Xiong, 2001; 2002). Zhu *et al.* (2006) found that wheat germ protein hydrolysates exhibited antioxidative activities. Hydrolysates from milk

protein can be used as natural antioxidants (Pihlanto, 2006). Sakanaka *et al.* (2004) found that egg yolk protein hydrolysates showed strong inhibitory activity on lipid oxidation in a linoleic acid model system as well as in beef and tuna homogenates (Sakanaka and Tachibana, 2006). Furthermore, antioxidants have been found in fish protein hydrolysate as well as fish processing by-products (Thiansilakul *et al.*, 2007a: Klompong *et al*., 2007, Je *et al*., 2005; Jun *et al*., 2004; Kim *et al*., 2001).

1.2.4 Protein hydrolysate

Protein hydrolysate is proteins that are broken down into peptides of varying sizes by chemical or enzymatical process. Protein hydrolysate has been reported as suitable sources of protein for human nutrition because of the balanced amino acid composition (Nolsøe and Undeland, 2009) and the better gastrointestinal absorption. Short chain peptides seem to be more advantageous than both intact protein and free amino acids (Clemente *et al*., 2000). Protein hydrolysates are used in products for special nutrition, such as diets for the elder and patients with impaired gastrointestinal absorption, hypoallergenic infant formulars, sports nutrition and weight-control diets as well as in consumer products for general use (Barca *et al*., 2000). Cleavage of proteins into smaller molecules or peptides during hydrolysis process could improve the functional and nutritional properties of food proteins (Kudo *et al*., 2009). The utilization of protein hydrolysates has increased significantly in the food industry (Shahidi *et al*., 1995). Hydrolysis process, therefore have been developed to convert such resources into marketable and acceptable forms (Gildberg, 1993). Protein hydrolysate can be produced from different protein sources. Its properties, functionality as well as stability are governed by the protease specificity, nature of protein substrate and degree of hydrolysis (Adler-Nissen, 1986). Acid proteases, even though are better for microbial growth prevention, have only low protein yield. Thus, milder enzymes at neutral and slightly alkaline condition have been used more frequently (Kristinsson and Rasco, 2000b).

1.2.4.1 Sources of protein hydrolysate

1.2.4.1.1 Plant protein hydrolysates

Plant proteins are extracted from abundant and renewable biological resources. In addition, their prices are generally lower than those of animal proteins (Nouri *et al*., 1997). Soybean has a high protein content with nutritionally balanced amino acid profile (Prakash *et al*., 2007) and also served as an inexpensive vegetable protein (Fan *et al*., 2005). Soy protein hydrolysates were wildly produced with the aid of enzyme. Flavourzyme and chymotrypsin were used for production of soy protein hydrolysate showing antioxidative activity in cooked meat pork patties (Peña-Ramos and Xiong, 2003). Tsou *et al*. (2010) used Flavourzyme to hydrolyze soy protein isolate in cooperation with ultrafiltration to obtain protein hydrolysate exhibiting anti-adipogenic activity. Seaweeds have been reported to contain protein as well as potential antioxidant compounds. Thus hydrolysis of this protein sources was performed to increase its antioxidative activity (Heo *et al*., 2005). Corn protein obtained as a byproduct of corn starch and oil production, which is rarely utilized in human foods as a food ingredient, was hydrolyzed with Alcalase and papain to gain corn protein hydrolysate, the value-added product (Kong and Xiong, 2006). The way to recovery of proteins from defatted wheat germ meal and to prepare acceptable products for human consumption is to produce wheat germ protein hydrolysate (Zhu *et al*., 2006). The wheat germ protein hydrolysate possessed both antioxidative and ACE inhibitory activity (Zhu *et al*., 2006; Matsui *et al*., 1999). Many plants used for oil making and were finally discarded as waste can be produced into protein hydrolysates with the aid of enzymes. Canola (Cumby *et al*., 2008) and peanut kernel (Kudo *et al*., 2009) are mainly used for its edible oil. A protein-rich meal after oil extraction were produced for protein hydrolysates with antioxidative activities using esperase and Pancreatin and Amano-P (Cumby *et al*., 2008; Kudo *et al*., 2009).

1.2.4.1.2 Milk protein hydrolysates

Milk is a complex mixture of proteins, lipids, carbohydrates, vitamins and minerals, thereby providing a complete diet for infants (Bayram *et al*., 2008). Whey represents the rich proteins with wide ranging nutritional, biological and

functional-food attributes (Ferreira *et al*., 2007). Pihlanto (2006) reported that the antioxidative activities of whey proteins can be increased through hydrolysis with certain enzymes. Whey protein isolate hydrolyzed with Flavourzyme or Protamex could retard the oxidation in cooked meat pork patties (Peña-Ramos and Xiong, 2003). Ferreira *et al*. (2007) conducted the tryptic hydrolysis of whey protein concentrate and found the ACE inhibitory activity in the resulting hydrolysate. Casein hydrolysate has been used as dairy ingredients in bread making for their nutritional benefits and functional properties including high volume and low firmness (Kenny *et al*., 2000). Sakanaka *et al*. (2005) reported that casein hydrolysates possessed strong antioxidative activity against *β*-carotene bleaching and also showed scavenging activity against free radicals. In general, casein hydrolysate contains the bitter peptides, leading to the less acceptability. Minagawa *et al*. (1989) found that aminopeptidase T was able to decrease and/or remove the bitterness of such a bitter peptide present in casein hydrolysates.

1.2.4.1.3 Fish protein hydrolysates

Lean fish species are ideal for fish protein hydrolysates preparation. Nevertheless, dark flesh species containing high amount of lipid, could be also used for production of protein hydrolysates (Klompong *et al*., 2007; Thiansilakul *et al*., 2007a). Additional treatments such as washing, defatting or centrifugation in order to remove the excess fat and pigments are required. The use of washed mince or surimi generally gives the high quality fish protein hydrolysates (Kristinsson and Rasco, 2000b). Therefore, protein source for hydrolysate production could obtain from both fish as well as their by-products.

- **Fish muscle protein hydrolysates**

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, 2000a), mackerel (*Scomber austriasicus*) (Wu *et al*., 2003), smooth hound (*Mustelus mustelus*) (Bougatef *et al*., 2009), yellow stripe trevally (*Selaroides leptolepis*) (Klompong *et*

al., 2007), red salmon (*Oncorhynchus nerka*) (Sathivel *et al*., 2005), round scad (*Decapterus maruadsi*) (Thiansilakul *et al*., 2007a), tilapia (*Oreochromis niloticus*) (Raghavan *et al*., 2008), silver carp (*Hypophthalmichthys molitrix*) (Dong *et al*., 2008) and loach (*Misgurnus anguillicaudatus*) (You *et al*., 2009). Recently, tuna dark muscle has been used for production of hydrolysate containing antioxidative peptides (Hsu, 2010). For some fish species, their myofibrillar proteins were isolated as protein isolate prior to enzymatic hydrolysis such as channel catfish (*Ictalurus punctatus*) (Theodore and other, 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. Moreover, oyster (*Crassostrea gigas*) protein (Qian *et al*., 2008b) and giant squid muscle (Rajapakse *et al*., 2005) was also used as a source of protein hydrolysate with the antioxidative activity.

- **Fishery by-products hydrolysates**

By-products obtained from fish processing plant have been used for the preparation of protein hydrolysate with functional properties and bioactivities. In general, a variety of proteases have been used to cleave the protein, resulting in the peptides with varying functional properties and bioactivity. By-products (heads, viscera, frames, skin, trimmings) of black scabbardfish (*Aphanopus carbo*) (Batista *et al*., 2010) and those (head and viscera) from sardinella (*Sardinella aurita*) (Bougatef *et al*., 2010b) 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) and Atlantic cod (*Gadus morhua*) (Šližyte *et al*., 2009) have been used for preparation of protein hydrolysate. Apart from solid byproducts, liquid effluent such as cooking juice from tuna (Jao and Ko, 2002; Hsu *et al*., 2009) have been used as the raw material for hydrolysate production.

Fish protein hydrolysates were also prepared from gelatin extracted from aquatic animals. Gelatin is a water soluble proteinaceous substance prepared by processes, which involve the destruction of the tertiary, secondary and to some extent the primary structure by thermal denaturation of native collagens. During the collagen

to gelatin transition, many non-covalent bonds are broken along with some covalent inter-and intramolecular bonds and a few peptide bonds. This results in the conversion of the helical collagen structure to a more amorphous form, known as gelatin (Figure 1) (Foegeding *et al.*, 1996). The properties of gelatin, molecular weight, number of amino acid residues and number of polypeptide chains depend on the position of the cleavages (Badii and Howell, 2006). Generally, the chemical composition of gelatin is similar to that of the parent collagen (Eastoe and Leach, 1977).

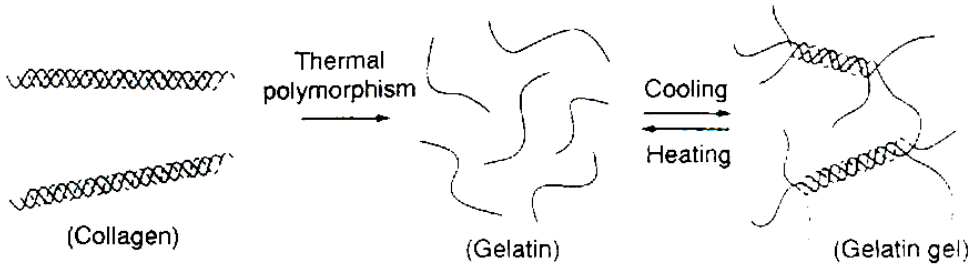

Figure 1. Conversion of collagen into gelatin.
Source: Okuzaki (2001)

Gelatin hydrolysates with the antioxidative activity have been produced from gelatin from the skin of Alaska pollack (Kim *et al*., 2001), hoki (Mendis *et al*., 2005b), cobia (Yang *et al*., 2008) and sole (Giménez *et al*., 2009). Furthermore, gelatin hydrolysates were also prepared from the skin of jumbo squid (Medis *et al*., 2005a), bullfrog (Qian *et al*., 2008a) and squid (Giménez *et al*., 2009). 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 Nile tilapia (*Oreochromis niloticus*) scale with the antioxidative activity was prepared by Ngo *et al*. (2010).

1.2.4.2 Enzymatic hydrolysis

Proteins cleaved into smaller molecules or peptides during enzymatic hydrolysis process could improve and upgrade the functional and nutritional properties of food proteins (Kudo *et al*., 2009). Hydrolysis process therefore have been developed to convert such resources into marketable and acceptable forms (Gildberg, 1993). 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 (Adler-Nissen, 1986). Proteases are used to obtain a more selective hydrolysis since they are specific for peptide bonds adjacent to certain amino acid residues (Peterson, 1978).

A wide variety of commercial enzymes has been used successfully to hydrolyze fish proteins (Table 2). Proteolytic enzymes from microorganisms such as Alcalase (Guerard *et al.,* 2001; Gbogouri *et al.*, 2004), Flavourzyme (Thiansilakul *et al.*, 2007a; Klompong *et al*., 2007), Neutrase (Šližyte *et al.*, 2005a; Phanturat *et al*., 2010), Protamex (Liaset *et al*., 2002; 2003) and Protease N (Wu *et al.*, 2003) are most suitable to prepare fish protein hydrolysates because of their high productivity. Commercial enzymes were also successfully used to produce protein hydrolysates with bioactivities. The antioxidative activities were found in protein hydrolysates derived from mackerel prepared by Protease N (Wu *et al.,* 2003). Sathivel *et al.* (2003) reported that herring by-products hydrolyzed by Alcalase showed the antioxidative activity. Protein hydrolysate from round scad and yellow stripe trevally hydrolyzed with Alcalase and Flavourzyme showed the antioxidative activities (Thiansilakul *et al*., 2007a; Klompong *et al*., 2007). Additionally, freshwater clam muscle hydrolysate prepared using Protomex, followed by Flavourzyme showed ACE inhibitory activity (Tsai *et al*., 2006)

Pancreatic enzymes also have been used for the production of protein hydrolysates (Table 2). Cod frame protein hydrolysates were prepared with crude proteinase extracted from tuna pyloric caeca. The hydrolysates possessed the effective antioxidative activity (Jeon *et al.,* 1999). Pepsin and mackerel intestinal crude enzymes were used for preparing yellowfin sole frame protein hydrolysates containing antioxidative peptides (Jun *et al.,* 2004). Antioxidative peptides were also

obtained in Alaska pollack frame protein hydrolysate prepared using crude proteinase from mackerel intestine (Je *et al.,* 2005). 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*., 2009)

However, the combination of proteinases includinge Alcalase, chymotrypsin, pancreatin and pepsin have also been used to increase the bioactivity of hydrolysates. Kim *et al.* (2007) employed six proteases (pepsin, trypsin, papain, α-chymotrypsin, Alcalase and Neutrase) for enzymatic hydrolysis to produce antioxidant peptides from hoki frame protein. Among hydrolysates, peptic hydrolysate had the highest antioxidant activity. Additionally, the combination of enzymes were used to produce protein hydrolysate with ACE inhibitory activity. Mullally *et al*. (1997) and Pihlanto *et al*. (1998) prepared ACE inhibitory whey protein hydrolysate using pepsin and trypsin. Byun and Kim (2001) produced ACE inhibitory peptides from Alaska pollack skin gelatin using the combination of Alcalase, pronase E and collagenase.

The selection of enzymes is usually based on a combination of efficacy and economics (Lahl, 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). Microbial proteinases commonly used include Alcalase, Flavourzyme, etc. However, fish trypsin have been reported as the potential source for the acceleration of protein hydrolysis (Kristinsson and Rasco, 2000c; Souza *et al.*, 2007). Fish trypsin has an advantage over commercial proteases due to the lower costs. The production of protein hydrolysate using fish trypsin or fish trypsin incombination with commercial ones therefore provide the bioactive hydrolysates with the lower production cost.

Table 2. Proteases and fish species used for production of protein hydrolysate

Proteases	Sources	References
Protamex	Atlantic salmon (*Salmo salar*, L.) frames	Liaset *et al*. (2002; 2003)
Thermolysin	Chum salmons (*Oncorhynchus keta*) muscle	Ono *et al*. (2003)
Protease N	Mackerel (*Scomber austriasicus*) meat	Wu *et al*. (2003)
Alcalase	Herring (*Clupea harengus*) head, gonad	Sathivel *et al*. (2003)
Alcalase	Atlantic salmon (*Salmo salar*) head	Gbogouri *et al*. (2004)
Flavourzyme, Neutrase	Cod (*Gadus morhua*) viscera, backbone	Šližyte *et al*. (2005a)
Alcalase	Red salmon (*Oncorhynchus nerka*) head	Sathivel *et al*. (2005)
Alcalase, Flavourzyme	Round scad (*Decapterus maruadsi*) mince	Thiansilakul *et al. (*2007a)
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)
Papain, Protamex	Loach (*Misgurnus anguillicaudatus*) meat	You *et al*. (2009)
Papain	Loach (*Misgurnus anguillicaudatus*) meat	You *et al*. (2010)
Orientase	Tuna (*Thunnus tonggol*) cooking juice	Hsu *et al*. (2009)
Tuna pyloric caeca proteases	Cod frame protein	Jeon *et al. (*1999)
Atlantic salmon proteases	Atlantic salmon (*Salmo salar*) muscle	Kristinsson and Rasco (2000c)
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)
Gastrointestinal proteases	Smooth hound (*Mustelus mustelus*) muscle	Bougatef *et al*. (2009)

1.2.4.3 Hydrolysis processes

Both chemical and biological methods are used for hydrolysis processes. In industry, chemical hydrolysis is commonly used due to the lower in cost, whereas biological processes using enzymes are employed for the high functionality and nutritive value products (Kristinsson and Rasco, 2000b). Prior to protein hydrolysis, some treatments, especially defatting or concentrating of the protein, have been employed to obtain the better quality of protein hydrolysate (Sikorski and Naczk, 1981). 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 protein 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. Phospholipid in the resulting mince might be also leached out during washing. In addition, washing not only removed sarcoplasmic proteins, but also concentrated the myofibrillar proteins (Baxter and Skonberg, 2008).

1.2.4.3.1 Chemical method

Chemical hydrolysis of protein is achieved by cleaving peptides bonds with either acid or base. However, it is relatively inexpensive and quite simple to conduct. There are many limitations to produce food ingredients since the process needs extreme temperatures, pHs and high pressure (harsh conditions). This process generally yields product with reduced nutritional qualities, poor functionality and restricted use as flavor enhancer (Kristinsson and Rasco, 2000b).

- **Acid hydrolysis**

Acid hydrolysis of proteins is used more commonly than hydrolysis under alkaline conditions. The acid hydrolysis is widely used to convert the under-utilized and secondary raw materials from fish into fertilizer due to the low production cost and the resulting extensive hydrolysis. Although the process is harsh

and hard to control, it is still the preferred method for hydrolyzing vegetable proteins (Kristinsson and Rasco, 2000b). For fish protein hydrolysate production, acid hydrolysis is carried out using hydrochloric acid or sulfuric acid. Hydrolysate products from this method need the neutralization after complete hydrolysis. Consequently, the resulting hydrolysate contains large amount of salt, which can interfere the functionality in food systems (Kristinsson and Rasco, 2000b). Furthermore, an essential amino acid, tryptophan, can be destroyed from this process. (Kristinsson and Rasco, 2000b).

- **Alkaline hydrolysis**

The use of alkaline reagent, generally sodium hydroxide, to hydrolyze protein often results in poor functionality and adversely affects the nutritive value of hydrolysate. Several toxic substances, e.g. lysinoalanine are formed during alkaline hydrolysis (Linder *et al*., 1996). Furthermore, the resulting hydrolysates have an inhibiting effect on proteolytic enzymes. Hence further hydrolysis using enzymes might reduce the rate of hydrolysis (Kristinsson and Rasco, 2000b).

1.2.4.3.2 Biochemical method

Food protein hydrolysates can be prepared by using proteolytic enzymes. The hydrolysis can be performed by both endogenous proteases already present in the fish viscera and muscle and by adding proteases from outer sources (Kristinsson and Rasco, 2000b). Compared with acid and alkaline hydrolysis, the enzymatic procedure can retain higher nutritional value.

-Autolytic hydrolysis

Autolytic hydrolysis, a simple operation, can be used in biochemical production of fish protein hydrolysate. Rate of hydrolysis depends on the action of the digestive enzymes of the fish itself. The end product of autolytic process is practically a fairly viscous liquid containing free amino acids and small peptides. The differences in fish species, seasons, types and amount of enzymes lead to the difficult to control the hydrolysis rate (Sikorski and Naczk, 1981). The application of autolytic hydrolysis is the production of fish sauces and fish silage (Kristinsson and Rasco, 2000b). Autolysis of fish promotes the hydrolysis process and lowers cost for

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 enzymes (Shahidi *et al*., 1995).

- Enzymatic hydrolysis

In order to modify the physicochemical, functional, and sensory properties of the native protein without threatening its nutritive value, enzymatic processes with added enzymes are used to hydrolyze food protein. These processes take place under mild circumstances and do not generate hydrolytic degradation products via racemization reactions occurred with acid and alkaline hydrolysis (Kristinsson and Rasco, 2000b). Enzymatic hydrolysis with added enzymes provides many advantages because it allows good control of the process with the desirable properties of resulting products (Kristinsson and Rasco, 2000b).

1.2.4.4 Hydrolysis conditions

Not only the specificities of enzymes, enzyme concentrations and reaction time significantly affected the hydrolysis process and the resulting hydrolysates (Sumaya-Martinez *et al*., 2005).

1.2.4.4.1 Enzyme concentration

Generally, the hydrolysis reaction is independent of the substrate concentration. Any change in the amount of product formed over a specified period of time is dependent upon the level of enzyme (Klompong *et al*., 2007). The relationship between activity and concentration is affected by many factors such as temperature, pH, etc. Guerard *et al*. (2001) suggested that the concentration of hydrolyzable bonds was one of the main variables controlling the hydrolysis rate. Cheftel *et al*. (1971) reported that an increase in enzyme concentration has a positive effect on overall proteolysis with subsequent increases in solubilization of fish protein concentrate. Protein hydrolysate from yellow stripe trevally showed the higher degree of

hydrolysis (DH) with increasing Alcalase concentration (Klompong *et al*., 2007) (Figure 2). However, the increasing ratio of enzyme concentrations did not yield the equal ratio of increasing DH.

1.2.4.4.2 Hydrolysis time

The enzymatic hydrolysis of protein is characterized by an initial rapid phase, during which a large of peptide bonds are hydrolyzed. Thereafter, the rate of enzymatic hydrolysis decreases and reaches a stationary phase where no apparent hydrolysis takes place (Shahidi *et al*., 1995). Klompong *et al*. (2007) reported that the hydrolysis curves of yellow stripe trevally using Alcalase and Flavourzyme showed high initial reaction rates, followed by decreases in the reaction rate up to the stationary phase (Figure 2). With increasing hydrolysis time, DH of silver carp protein hydrolyzed by Alcalase and Flavourzyme increased rapidly in 15 min and slowed down thereafter (Dong *et al*., 2008). The similar result was also reported by Giménez *et al*. (2009) for the hydrolysis of skin from sole and squid with Alcalase. The high initial rate observed indicates that the maximum cleavage of peptides occurred.

1.2.4.5 Functional properties of protein hydrolysates

Controlled hydrolysis can provide and improve functional properties of protein (Klompong, *et al*., 2007). The functional properties of proteins are those physicochemical properties that govern their performance and behavior in food systems during their preparation, processing, storage and consumption and also influence the use of protein hydrolysate as an ingredient in food (Sathivel *et al*., 2005). Factors affecting the functional properties of food proteins have been classified as intrinsic factors and extrinsic factors (Panyam and Kilara, 1996). Most of the extrinsic factors are dictated by the food system, the processing and the storage conditions. The intrinsic factors are entirely characteristic of the protein, which were able to modify mainly by hydrolysis (Panyam and Kilara, 1996). Adler-Nissen (1986) and Kristinsson and Rasco (2000b) reported that hydrolysis process directly influences the molecular size, hydrophobicity and polar group of the hydrolysate.

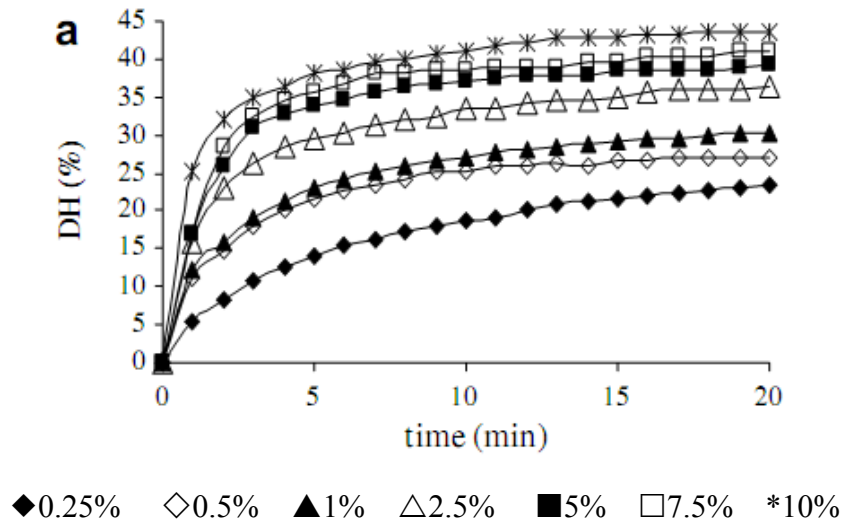

Figure 2. Effect of different concentrations of Alcalase on DH of yellow stripe trevally mince. The hydrolysis reaction was run for 20 min at pH 8.5 and 60°C. The pH of mixture was maintained constant during hydrolysis using pH stat method.

Source: Klompong *et al*. (2007)

Functional properties of protein were affected directly by the characteristic of protein hydrolysates obtained (Klompong *et al*., 2007; Limam *et al*., 2008)

1.2.4.5.1 Solubility

Solubility is one of the most important pre-requisite properties of protein. In many protein-based formulations, such as emulsions and foams, good solubility for the protein is usually required (Yin *et al*., 2008). The improvement in protein solubility by the enzymatic hydrolysis was attributed to the release of soluble peptides by the cleavage of proteins into smaller peptide units and the increased number of exposed amino and carboxyl groups (Yin *et al*., 2008). Protein hydrolysates have been useful because of their high solubility over a wide pH range and during heat treatment (Barca *et al*., 2000). Generally, the pH levels near the pI of muscle proteins (around pH 4.0-5.0) gave the lowest solubility (Mazorra-Manzano *et al*., 2010).

Conversely, as the pH was adjusted away from the pI of proteins, the protein net charge increased, thus promoting molecular repulsion. Good solubility of hydrolyzed protein over a broad pH range is very interesting for food applications since many functional properties of proteins depend upon their capacity to initially go into solution. The excellent solubility of the protein hydrolysate suggests that they may have many potential applications in formulated food systems (Pacheco-Aguilar *et al*., 2008; Santos *et al*., 2009; Foh *et al*., 2010; Mazorra-Manzano *et al*., 2010). A high correlation between solubility and the DH has been reported. However, extensive hydrolysis could have a negative impact on other functional properties (Kristinsson and Rasco, 2000b).

Giménez *et al*. (2009) studied the functional properties of gelatin hydrolysates from sole and squid skin gelatin prepared using Alcalase. The DH of 35 and 50% of sole and squid skin gelatin hydrolysates had the solubility higher around 98%. Solubility of jumbo squid protein hydrolysate decreased as temperature increased up to 50°C due to the aggregation of protein. At pH around 5, the lowest solubility was observed (Fuente-Betancourt *et al*., 2009). Šližyte *et al*. (2009) suggested that fish protein hydrolysates had good solubility over a wide pH and ionic strength range.

1.2.4.5.2 Emulsifying property

Generally, hydrolysates are surface-active materials and can stabilize the oil-in-water emulsions because of their exposed hydrophilic and hydrophobic groups (Kristinsson and Rasco, 2000b). Thus, the formation of the aggregates in the hydrolysates might inhibit the formation of a viscoelastic membrane on the oil-in-water surface, leading to the increase oil droplets coalescence (Gbogouri *et al*., 2004; Yin *et al*., 2008). Emulsifying property was affected by the solubility (Pacheco-Aguilar *et al*., 2008). The high solubility of hydrolysates generates the low molecular weight peptides which rapidly diffuse to and absorb at the interface. However, these small peptides are less efficient in reducing the interfacial tension due to lack of unfolding and reorientation at the interface as large peptides do (Gbogouri *et al*., 2004). This leads to the rapid flocculation and the loss of protein layer stability (Choi

et al., 2009). The molecular weight distributions of the hydrolysates play a key role in stabilizing emulsions. The optimal molecular size or chain length for peptides provided good foaming and emulsifying properties, while extensive hydrolysis with small peptides reduced these properties (Šližyte *et al*., 2009; Mazorra-Manzano *et al*., 2010). Peptide with a minimum length of about 20 residues is required to possess good emulsifying and interfacial properties (Limam *et al*., 2008; Choi *et al*., 2009). Furthermore, Santos *et al*. (2009) and Šližyte *et al*. (2009) suggested that the differences in emulsifying properties of protein hydrolysates were probably due to the amino acid compositon and sequence.

Gelatin hydrolysates from sole and squid skin gelatin showed a decrease in emulsifying activity index (*EAI*) with increasing protein concentration (Giménez *et al*., 2009). This was due to an increase in interactions between proteins with increasing concentration, thus resulting in a lower protein concentration at the oil-water interface. However, emulsion stability index (*ESI*) showed no differences with all protein concentrations. The temperature also affects emulsifying property. Fuente-Betancourt *et al*. (2009) reported that *EAI* of jumbo squid protein hydrolysate continuously decreased with increasing temperature. The lower *EAI* was the result of the loss in solubility caused by protein aggregation. However, Choi *et al*. (2009) reported that *EAI* of croaker hydrolysate did not correlate with solubility. It was governed by protease type and DH. Pacheco-Aguilar *et al*. (2008) found that among pHs tested, pH 10 provided the Pacific whiting muscle hydrolysate with higher *EAI*. This was resulted from the negative charges generated, leading to the repulsion between proteins and a better orientation at the oil-water interface.

1.2.4.5.3 Foaming property

Foams are of particular interest because they provide desirable textures to many aerated foods, such as ice cream, whipped topping, breads, cakes, meringues, beers, champagne, cheese, butter, spreads, confectionary, sausages, etc. (Ruíz-Henestrosa *et al*., 2009). Protein hydrolysates possess the foaming property due to their excellent surface property. Yin *et al*. (2008) and Pacheco-Aguilar *et al*. (2008) suggested that factors mainly contributing to foaming capacity are viscosity, surface

hydrophobicity and solubility. Foaming characteristics includes the effectiveness of gas encapsulation (foaming capacity) and the life time of the foam produced (foaming stability). The basic requirement for a protein to be a good foaming agent is the ability to be rapidly adsorbed at the air-water interface and undergo rapid rearrangement at the interface as well as the formation of a cohesive viscoelastic film via intermolecular interaction. A protein may have excellent foaming capacity but it may not necessarily produce a stable foam (Mazorra-Manzano et al., 2010). The decreases in chain length of peptides as a result of enzymatic hydrolysis may mainly account for the decreases in foaming capacity and foaming stability (Yin *et al*., 2008). However, Limam *et al*. (2008) reported the opposite aspect that the production of smaller molecular sizes peptides, resulting in higher foaming properties. The pH of the dispersing medium also dramatically influences foaming properties, especially foam stability. Foaming characteristic was highest when pH was close to the isoelectric point of protein (Kinsella, 1976).

Foaming ability of sole and squid gelatin hydrolysates increased with increasing concentration due to an increase in the rate of diffusion. However, after 30 min whipping, the slight differences in foam stability were observed at increasing hydrolysates concentration (Giménez *et al*., 2009). The high foam stability resulted from the high degree of some amino acid residues in protein structure, e.g. proline. lysine, which leads to a higher ability to establish protein-protein interactions via hydrogen bonds, resulting in a denser network that favors foam stabilization (Giménez *et al*., 2009). The decrease in the foaming stability is probably due to the lower surfactant activity of smaller peptide chains from extensive hydrolysis (Foh *et al*., 2010). Fuente-Betancourt *et al*. (2009) reported the sharp increase in foaming ability as temperature increased up to 50°C. This was due to the hydrophobic interaction between proteins caused by heating, thereby rendering the dense peptide surfactant.

1.2.5 Production of fish protein hydrolysates containing bioactive peptides

Many dietary proteins from marine sources possess specific biological properties after proteolytic hydrolysis using various proteases (Amarowicz and Shahidi, 1997; Kristinsson and Rasco, 2000b; Wu *et al*., 2003; Sathivel *et al*., 2003; Klompong *et al*., 2007; Thiansilakul *et al*., 2007a; Qian *et al*., 2008b). Fish protein hydrolysates have a potential to be protein supplements in cereal foods and soups (Venugopal and Shahidi, 1995). In some countries, fish protein hydrolysates are used as a milk substitute and as flavoring compounds (Stephens *et al*., 1976). Therefore, protein hydrolysates, especially fish protein hydrolysates from enzymatic hydrolysis, are the best among protein hydrolysates, in term of nutritional properties, balanced amino acid composition and high digestibility (Kristinsson and Rasco, 2000b).

Enzymatic hydrolysis has been widely used to prepare the hydrolysate with the bioactivity. To obtain the hydrolysate with high stability with less pro-oxidant, the raw materials can be washed to remove heme protein and lipids (Raghavan *et al*., 2008). Additionally, the antioxidants can be added before hydrolysis. To enhance the hydrolysis process, the raw materials are subjected to size reduction such as by homogenization. Bone, scales or skin, which may interfere with enzymatic hydrolysis, should be removed. Thereafter, the selected protease is added into the mixture containing the protein substrate, which is previously adjusted to the desired pH value. Different proteases exhibited the varying specificity and reaction rate in hydrolysis of peptide chains, resulting the different resulting 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 produced had MW of 1.7 kDa. To maximize autolysis or hydrolysis, optimal pH and temperature are implemented, thereby enhancing the cleavage of peptides. Nevertheless, the autolysis or the use of endogenous protease might be of difficulty in controlling the degree of hydrolysis or obtaining the desired peptides. After the hydrolysis, the reaction is terminated by inactivation of protease by heat treatment or pH adjustment. The combination of pH and temperature to denature the protease used is another approach to avoid the harsh condition, which may affect the resulting hydrolysate or peptides. The reaction mixture containing the peptides as well as the

unhydrolyzed debris is centrifuged or filtered. The supernantant or filtrate is concentrated or dried. The hydrolysate can be an excellent source of peptides with functionalities and bioactivities, which are determined by the types of protease, pretreatment of raw materials, the condition of hydrolysis, etc.

To produce protein hydrolysate with antioxidative activities, the types of proteases have the influence on the peptides produced and their activities (Table 3). 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). Tuna cooking juice was hydrolyzed using Protease XXIII from *Aspergillus oryzae* and the hydrolysate obtained displayed the DPPH radical scavenging ability (Jao and Ko, 2002). Jun *et al*. (2004) produced protein hydrolysate from yellowfin sole frame using pepsin and mackerel intestine crude enzyme, the hydrolysate showed a strong antioxidative activity in a linoleic acid model system. 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). Kim *et al*. (2007) prepared antioxidative peptide from hoki frame protein hydrolysate 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. Oyster protein digested in gastrointestinal model system showed the enhanced antioxidative activity (Qian *et al*., 2008b). 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.

1.2.6 Antioxidative peptides from fish protein hydrolysates

Numerous peptides from fish protein hydrolysate have been purified and identified for their amino acid sequences and molecular weights. 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

Table 3. Source, amino acid sequence and molecular weight of antioxidative peptides from some aquatic 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 fram	Pepsin, Mackerel intestine crude enzyme	1300	Arg-Pro-Asp-Phe-Asp-Leu-Glu-Pro-Pro-Tyr	Jun *et al*. (2004)
Alaska Pollack fram	Mackerel intestine crude enzyme	672	Leu-Pro-His-Ser-Gly-Tyr	Je *et al*. (2005)
Giant squid muscle	Trypsin	747	Asn-Gly-Leu-Glu-Gly-Leu-Lys,	Rajapakse *et al*. (2005)
		1307	Asn-Ala-Asp-Phe-Gly-Leu-Asn-Gly-Leu-Glu-Gly-Leu-Ala	
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)
Tuna backbone	Pepsin	1519	Val-Lys-Ala-Gly-Phe-Ala-Trp-Thr-Ala-Asn-Gln-Gln-Leu-Ser	Je *et al*. (2007)
Hoki fram	Pepsin	1801	Glu-Ser-Thr-Val-Pro-Glu-Arg-Thr-His-Pro-Ala-Cys-Pro-Asp-Phe-Asn	Kim *et al*. (2007)
Oyster protein	Pepsin	1600	Leu-Lys-Gln-Glu-Leu-Glu-Asp-Leu-Leu-Glu-Lys-Gln-Glu	Qian *et al*. (2008)
Bigeye tuna dark muscle	Pepsin	1222	Leu-Asn-Leu-Pro-Thr-Ala-Val-Tyr-Met-Val-Thr	Je et al. (2008)
Tuna cooking juice	Orientase	1305	Pro-Val-Ser-His-Asp-His-Ala-Pro-Glu-Tyr	Hsu *et al*. (2009)
		938	Pro-Ser-Asp-His-Asp-His-Glu	
		584	Val-His-Asp-Tyr	
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*. (2010)

implemented such as the use of multi-step of hydrolysis with different protease (Phanturat *et al*., 2010). Also, types of proteases used also showed the impact on the bioactivity of peptides formed (Klompong *et al*., 2007; Phanturat *et al.,* 2010).

Different radical scavenging activity is not determined by only amino acid composition but also special amino acid sequences (Kim *et al*., 2001; Mendis *et al*., 2005b). Antioxidant peptides derived from different sources have exhibited varying potencies to scavenge free radicals. The exact mechanism of scavenging these radicals by the peptide is not clearly understood. 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). Park *et al*. (2001) suggested that the quenching of free radicals by natural antioxidants has been reported through donation of hydrogen. Jao and Ko (2002) reported that hydrolysate of tuna cooking juice contained several amino acids, such as tyrosine, methionine, histidine, tryptophan, lysine and proline, which function as antioxidants. Dávalos *et al*. (2004) indicated that tryptophan, tyrosine and methionine showed the highest antioxidant activity among the amino acids, and followed by cysteine, histidine and phenylalanine, while histidine and hydrophobic amino acids was suggested by Peña-Ramos *et al*. (2004) as the key factor in delay lipid oxidation. 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. Wang *et al*. (2008) stated that peptides exhibited good antioxidative activity usually contain certain amino acids such as histidine, proline, tyrosine and lysine. Bougatef *et al.* (2010b) indicated that peptides containing histidine, tryptophan and tyrosine residues possessed antioxidative 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. Moreover, Suetsuna *et al*. (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 once it is located in the C-terminus end of the sequence (Suetsuna *et al*., 2000).

In general, peptide containing tyrosine usually exhibited a strong free radical scavenging activity due to the phenolic hydroxyl groups of tyrosine residue, which contribute substantially to scavenging activity toward free radicals via the mechanism of donating a hydrogen atom from their hydroxyl group (Suetsuna *et al*., 2000; Guo *et al.,* 2009). Other aromatic amino acids, tryptophan and phenylalanine, are generally considered as effective radical scavengers, because they can donate protons easily to electron deficient radicals while at the same time maintaining their stability via resonance structures (Rajapakse *et al*., 2005; Zhang *et al*., 2009). In case of histidine-containing peptides, such as carnosine, anserine, and balenine (also known as ophidine), the noticeable chelating activity was attributed to the proton-donation ability of imidazole group of histidine (Mendis *et al*., 2005b). Hydrophobic peptides can help scavenging free radicals by keeping close contact with oxidizing substance, leading to the rapid scavenging of radicals (Mendis *et al*., 2005b).

1.2.7 Angiotensin-I Converting enzyme (ACE) inhibitory peptides from fish protein hydrolysate

Angiotensin-I converting enzyme (ACE; EC 3.4.15.1) is a nonspecific dipeptidyl carboxypeptidase (Vermeirssen *et al*., 2002) with a high molecular mass of 146 kDa. ACE is widely distributed in mammalian tissues, predominantly as a membrane-bound exoenzyme in vascular endothelial cells and also in several other cell types including absorptive epithelial, neuroepithelial, and male germinal cells (Li *et al*., 2004). This enzyme plays a key physiological role in the blood pressure regulating rennin-angiotensin system, kallikrein-kinin (Li *et al*., 2004; Pihlanto, 2001) and also immune system (Pihlanto, 2001) as shown in diagram in Figure 3.

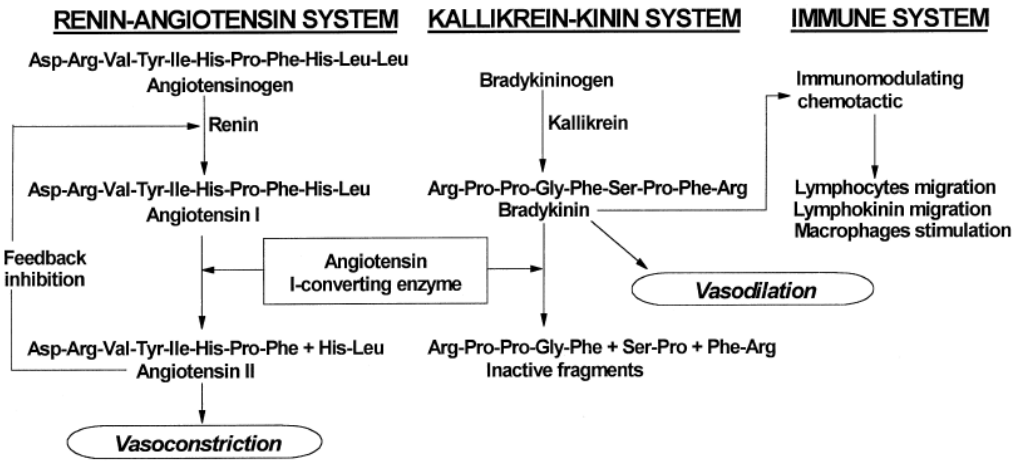

Figure 3. Role of ACE in rennin-angiotensin, kallikrein-kinin and immune system
Source: Pihlanto (2001)

ACE cleaves dipeptide (histidyl-leucine) from the C-terminus of the inactive decapeptide angiotensin I into the potent vasoconstricting octapeptide angiotensin II (Lee *et al*., 2010). This potent vasoconstrictor is also involved in the release of a sodium-retaining steroid, aldosterone, from the adrenal cortex, which has a tendency to increase blood pressure (Li *et al*., 2004). This enzyme is also a potent pressor hormone, and inactivates the vasodilating nonapeptide bradykinin (Chiang *et al*., 2006), a mediator of inflammation and vasodilator peptide which is also a potent stimulator of vasodilator prostaglandin and nitric oxide synthesis. (Meng and Oparil, 1996). Converting of angiotensin I causes the increasing in blood pressure that leads to hypertension which has been considered the most common serious chronic health problem and related to the incidence of circulatory organ diseases, including arteriosclerosis, stroke, myocardial infarction and also end-stage renal disease (Jung *et al*., 2006a, Tsai *et al.*, 2006, Chiang *et al*., 2006, and Itou and Akahane, 2004). Inhibition of ACE is considered to be a useful therapeutic approach in the treatment of hypertension. Therefore, in the development of drugs to control high blood pressure, ACE inhibition has become an important target (Lo *et al.*, 2006).

There are many synthetic ACE inhibitors, based on the dipeptide configuration. The synthetic ACE inhibitors are one group of drugs currently used as effective antihypertensive prodrugs, such as captopril, enalapril, alacepril, benazepril,

and lisinopril (Ono *et al*., 2006, Jung *et al*., 2006a, and Vermeirssen *et al.*, 2002). Unfortunately, these synthetic ACE inhibitors can cause many significant undesirable side effects including a persistent dry cough (Lo *et al*., 2006), taste disturbances, and skin rash (Kim *et al*., 2003). Therefore, the search for alternative substance with safer and naturally desirable for prevention of hypertension instead of synthetic one has been focused.

A number of peptides have shown their ACE inhibitory activity. Lee *et al*. (2010) indicated that the natural hypotensive ACE inhibitors are peptides, because of their high competitive affinity with the ACE active site. Mullally *et al*. (1997) reported ACE inhibitory activity of whey protein hydrolysate. Antihypertensive peptide was isolated from egg white hydrolysate (Miguel *et al*., 2007). Nakashima *et al*. (2002) found that peptides of porcine skeletal muscle proteins hydrolysate showed antihypertensive activities. Hydrolysate from whey protein gave higher ACE inhibitory activity than pea protein hydrolysate (Vermeirssen *et al*., 2002).

ACE inhibitory peptides were also isolated from fish protein hydrolysate such as salmon protein hydrolysate (Ewart *et al*., 2009), chum salmon protein hydrolysate (Ono *et al*., 2006), Atlantic salmon, Coho salmon, Alaska Pollack, Southern blue whiting protein hydrolysates (Nakajima *et al*., 2009) and tilapia protein hydrolysate (Raghavan and Kristinsson 2009). Additionally, fish by-products such Alaska pollack skin (Byun and Kim, 2001), the fermented mackerel (Itou and Akahane, 2004), yellowfin sole fram (Jung *et al*., 2006a), tuna cooking juice (Ko *et al*., 2006), sardinelle by-products (Bougatef *et al*., 2008b) and tuna frame protein hydrolysate (Lee *et al*., 2010) were reported to possess ACE inhibitory activity. ACE inhibitory peptides from aquatic protein hydrolysates are summarized in Table 4.

1.2.8 Calcium binding peptides

Calcium (Ca) is an important mineral for human body. Generally, the essential source of calcium is milk and other diary products (Anderson and Garner, 1996). Casein phosphopeptides (CPP) obtained after intestinal digestion of casein enhance bone clarification (Jung *et al*., 2006b). CPP are able to bind Ca and prevent

Table 4. Source and amino acid sequence of ACE inhibitory peptides from some aquatic protein hydrolysates**.**

Sources	Amino acid sequences	References
Alaska pollack skin	Gly-Pro-Leu, Gly-Pro-Me	Byun and Kim (2001)
Pelagic thresher muscle	Val-Trp, Met-Trp, Ile-Lys-Trp, Leu-Trp-Ala, Val-Ser-Trp, Val-Thr-Arg, Phe-Arg-Val-Phe-Thr-Pro-Asn	Nomura *et al*. (2002)
Sea bream scales	Gly-Tyr,Val-Tyr,Gly-Phe, Val-Ile-Tyr.	Fahmi *et al*. (2004)
Yellowfin sole frame protein	Met-Ile-Phe-Pro-Gly-Ala-Gly-Gly-Pro-Glu-Leu	Jung *et al*. (2006a)
Salmon muscle	Phe-Leu	Ono *et al*. (2006)
Salmon muscle	Phe-Ile-Ala, Ile-Val-Phe	Enari *et al*. (2008)
Shark meat	Cys-Phe, Glu-Tyr, Met-Phe, Phe-Glu, Cys-Phe, Glu-Tyr, Phe-Glu	Wu *et al*. (2008)
Salmon protein	Val-Leu-Trp, Val-Phe-Tyr, and Leu-Ala-Phe.	Ewart *et al*. (2009)
Tuna frame protein	Gly-Asp-Leu-Gly-Lys-Thr-Thr-Thr-Val-Ser-Asn-Trp-Ser-Pro-Pro-Lys-Try-Lys-Asp-Thr-Pro	Lee *et al*. (2010)

the precipitation of Ca phosphate salts, resulting in the increased soluble Ca availability for absorption (Yuan and Kitts, 1994). However, some groups of people do not drink milk caused by the lactose indigestion and intolerance. Therefore, the peptides from other sources, especially from aquatic source can be an alternative for the supplement in food to increase Ca solubility and bioavailability. Bone oligopeptides with high affinity to calcium was prepared from hoki bone with the aid of tuna intestine crude enzyme, which was able to degrade hoki bone matrices comprising collagen, non-collageneous proteins, carbohydrate and minerals (Jung *et al*., 2005). Fish bone phosphopeptides (FBP) containing 23.6% phosphorus had the molecular weight of 3.5 kDa could solubilize the calcium (Jung *et al*., 2005). Fish bone peptide II (FBP II) with high ratio of phosphopeptide from hoki bone could inhibit the formation of insoluble Ca salts. The levels of femoral total Ca, bone mineral density and strength were increased in ovariectomised rats fed with FBP II diet (Jung *et al*., 2006b). Alaska pollack backbone peptide, Val-Leu-Ser-Gly-Gly-Thr-Thr-Met-Ala-Met-Ala-Met-Tyr-Thr-Leu-Val with the MW of 1,442 Da, prepared

using pepsin hydrolysis showed the affinity to calcium ion on the surface of hydroxyapatite crystals. The peptide could solubilize the similar content of calcium to casein phosphopeptide (Jung *et al*., 2006c). Furthermore, calcium binding peptide derived from pepsinolytic hydrolysate of hoki frame was identified as Val-Leu-Ser-Gly-Gly-Thr-Thr-Met-Tyr-Ala-Ser-Leu-Tyr-Ala-Glu with MW of 1,561 Da (Jung and Kim, 2007). Therefore, fish bone oligophosphopeptide could be used as the nutraceutical to increase the absorption of calcium.

1.3 Objectives

1. To comparatively study the proteolytic activity of the extract from fish pyloric caeca obtained from surimi processing plants, including brownstripe red snapper, bigeye snapper and threadfin bream and to produce hydrolysate with antioxidative acitivity from fish skin gelatin using proteases from pyloric caeca of those fish.
2. To purify and characterize trypsin from pyloric caeca of brownstripe red snapper.
3. To elucidate the effect of washing and membrane separation on the removal of pro-oxidant and membranes and to determine the antioxidative activity of protein hydrolysates from pretreated mince of brownstripe red snapper prepared using proteases from its pyloric caeca.
4. To obtain antioxidative protein hydrolysate prepared from brownstripe red snapper muscle with the aid of its pyloric caeca protease and commercial proteases using two-step hydrolysis process and to study their antioxidative activities and ACE inhibitory activity as well as the antioxidative stabilities in different oxidation models.
5. To isolate and identify antioxidative peptides from protein hydrolysate of brownstripe red snapper muscle produced by two-step hydrolysis process.
6. To study functional properties and antioxidative activities of protein hydrolysate from brownstripe red snapper muscle prepared by two-step hydrolysis process and to investigate the stability of protein hydrolysate during extended storage.

CHAPTER 2

COMPARATIVE STUDY ON THE PROTEASES FROM FISH PYLORIC CAECA AND THE USE FOR PRODUCTION OF GELATIN HYDROLYSATE WITH ANTIOXIDATIVE ACTIVITY

2.1 Abstract

Proteases from pyloric caeca extract of three fish species including brownstripe red snapper (*Lutjanus vitta*), bigeye snapper (*Priacanthus tayenus)* and threadfin bream (*Nemipterus marginatus*) were comparatively studied. The extracts from bigeye snapper and threadfin bream exhibited the highest hydrolytic activities toward casein, α-*N*-benzoyl-DL-arginine-*p*-nitroanilide and α-*N*-*ρ*-toluene-sulfonyl-L-arginine methyl ester at pH 8.0 and 60^{o}C and pH 8.5 and 55^{o}C, respectively. The extract of brownstripe red snapper showed the optimal pH and temperature of 8.0 and 60^{o}C with all substrates used except the optimal temperature was 65^{o}C when casein was used. All proteases were strongly inhibited by soybean trypsin inhibitor (SBTI) and *N*-*ρ*-tosyl-L-lysine chloromethylketone (TLCK) and partially inhibited by *N*-tosyl-L-phenylalanine chloromethylketone for all substrates tested, suggesting that trypsin-like proteases were the major enzymes. Substrate-gel activity staining of 40-60% ammonium sulfate (AS) fraction revealed that major activity bands were observed with molecular weight of 24, 22 and 20 kDa for brownstripe red snapper, bigeye snapper and threadfin bream, respectively. Those activity bands were partially inhibited by SBTI and TLCK. AS fraction was further used to produce fish skin gelatin hydrolysates with different degrees of hydrolysis (DH). Hydrolysate with DH of 15% exhibited the highest DPPH and ABTS radical scavenging activities and ferric reducing antioxidant power ($p < 0.05$). Therefore, the extract from pyloric caeca could be used to produce the gelatin hydrolysates possessing antioxidative activities.

2.2 Introduction

Enzymatic hydrolysis is widely applied to improve and upgrade the functional and nutritional properties of food proteins (Klompong, *et al*., 2007). Proteases from different sources are commonly used to obtain a more selective hydrolysis since they are specific for peptide bonds adjacent to certain amino acid residues (Peterson and Johnson, 1978). Fish viscera generated during the processing contain a variety of enzymes including proteases. Proteases are potential enzymes for industrial applications and could produce the new bioactive molecules (Shahidi and Kamil, 2001). Numerous peptides derived from hydrolyzed food proteins have been shown to have antioxidative activities. Fish protein hydrolysate such as skin gelatin hydrolysate from Alaska pollack (Kim *et al*., 2001) or frame protein hydrolysate from cod (Jeon *et al*., 1999), yellowfin sole (Jun *et al*., 2004) and Alaska pollack (Je *et al*., 2005) have been reported to exhibit antioxidative activity.

Brownstripe red snapper (*Lutjanus vitta*), bigeye snapper (*Priacanthus macracanthus*) and threadfin bream (*Nemipterus marginatus)* are the potential raw materials for surimi production in Thailand (Jongjareonrak, *et al*., 2006; Benjakul, *et al*., 2001; Yongswawatdigul and Park, 2002). Processing wastes generated during surimi processing, especially skin and viscera can be used as raw material for gelatin production and the essential source of proteases, respectively.

Trypsin (EC 3.4.21.4) is a proteolytic enzyme in the group of serine proteinases and is more selective than any other enzyme, cleaving peptide bonds at the carboxyl terminus of lysine and arginine residues exclusively (Bernhard, 1968). Trypsin consists of single peptide chain with molecular weight (MW) of typically 24 kDa (Torrissen and Male, 2000). This enzyme has been isolated from pyloric caeca or intestines of various fish. Trypsin with MW of 24 kDa was purified from pyloric caeca of arabesque greenling (*Pleuroprammus azonus*) (Kishimura *et al*., 2006), jacopever (*Sebastes schlegelii*), elkhorn sculpin (*Alcichthys alcicornis*) (Kishimura *et al*., 2007) and walleye pollock (*Theragra chalcogramma*) (Kishimura *et al*., 2008). The MW of purified trypsin from pyloric caeca of Monterey sardine (*Sardinops sagax caerulea*) (Castillo-Yañez *et al*., 2005) and New Zealand hoki (*Macruronus*

Novaezealandlae) (Shi *et al*., 2007) were estimated to be 25 and 26 kDa, respectively, while trypsins with MW of 28 kDa were obtained from pyloric caeca of bluefish (*Pomatomus saltatrix*) (Klomklao *et al*., 2007d) and chinook salmon (*Oncorhynchus tshawytscha*) (Kurtovic *et al*., 2006). Trypsins with MW of 24 kDa were also purified from viscera of Japanese anchovy (*Engraulis japonica*) (Kishimura *et al*., 2005) and true sardine (*Sardinops melanostictus*) (Kishimura *et al*., 2006). Furthermore, spleen of skipjack tuna (*Katsuwonus pelamis*) was reported to contain trypsin with MW of 24 kDa (Klomklao *et al*., 2007b).

Recovery of proteolytic enzymes, especially trypsin, from fish viscera is a promising approach to minimize the economics and ecological problem of this processing waste. Additionally, gelatin hydrolysate with neutraceutical property from fish skin produced by the aid of fish proteases can be obtained as the new value-added product with high market value. The objectives of this study were to study the proteolytic activity of the extract from fish pyloric caeca obtained from surimi processing plants and to produce hydrolysate with antioxidative acitivity from fish skin gelatin using proteases from fish pyloric caeca.

2.3 Materials and Methods

2.3.1 Chemicals

β-mercaptoethanol (*β*ME), casein, tyrosine, α-*N*-benzoyl-DL-arginine-*p*-nitroanilide (BAPNA), α-*N*-*ρ*-toluene-sulfonyl-L-arginine methyl ester (TAME), 1-(L-trans-epoxysuccinyleucylamino)-4-guanidinobutane (E-64), iodoacetic acid, soybean trypsin inhibitor (SBTI), *N*-*ρ*-tosyl-L-lysine chloromethylketone (TLCK), *N*-tosyl-L-phenylalanine chloromethylketone (TPCK), ethylenediaminetetraacetic acid (EDTA), pepstatin A, 2,2′-azinobis(3-ethylbenzothiazoline-6-sulphonic acid) (ABTS), 1,1-diphenyl-2-picrylhydrazyl (DPPH), 2, 4, 6-tripyridyl-triazine (TPTZ), 2,4,6-trinitrobenzenesulfonic acid (TNBS) and Trolox were purchased from Sigma Chemical Co. (St. Louis, MO, USA). Tris (hydroxymethyl) aminomethane (Tris-HCl), trichloroacetic acid (TCA) and ferric chloride were obtained from Merck

(Darmstadt, Germany) and sodium sulfite was obtained from Riedel-deHaën (Seelze, Germany). All chemicals were of analytical grade.

2.3.2 Collection of fish pyloric caeca

Viscera from brownstripe red snapper (*Lutjanus vitta*), bigeye snapper (*Priacanthus tayenus)* and threadfin bream (*Nemipterus marginatus*) were collected from a surimi processing plant in Songkhla province, Thailand. Viscera were placed in polyethylene bag, stored in ice with a fish/ice ratio of 1:2 (w/w) and transported to Department of Food Technology, Prince of Songkla University. Upon arrival, viscera were excised and only pyloric caeca were selected. Pyloric caeca obtained were packaged in polyethylene bag, immediately frozen and stored at -20°C until use.

2.3.3 Preparation of pyloric caeca extract

Frozen pyloric caeca was thawed using running water until the core temperature reached -2 to 0°C. Then the sample was cut into small pieces (1-1.5 cm in thickness) and was finely ground into powder in liquid nitrogen according to the method of Hau and Benjakul (2006). Pyloric caeca powder was suspended in extraction buffer (50 mM Tris-HCl buffer, pH 8.0 containing 10 mM $CaCl_2$) with a ratio of 1:10 (w/v). The mixture was homogenized for 2 min using a homogenizer (IKA labortechnik, Selangor, Malaysia) at a speed of 11,000 rpm. The homogenate was continuously stirred at 4°C for 30 min, followed by centrifugation at 11,200 x *g* for 30 min at 4°C to remove the tissue debris using a refrigerated centrifuge (Sorvall, RC-B Plus, Newtown, CT, USA). The supernatant was collected and filtered through a Whatman paper No. 1 (Whatman International Ltd., Maidstone, England). The filtrate obtained was referred to as ‘pyloric caeca extract, PCE’. PCE was kept in ice and used within 2 days.

2.3.4 Activity assays

2.3.4.1 Proteolytic activity assay

Proteolytic activity of PCE was determined using casein as a substrate according to the method of Klomklao *et al.* (2004) with a slight modification. To initiate the reaction, 200 µL of sample were added into assay mixtures containing 200 µL of 2% casein (w/v), 200 µL of distilled water and 625 µL of reaction buffer containing 10 mM $CaCl_2$. The reaction was conducted under the selected condition for 15 min. A 200 µL of 50% (w/v) TCA was added to terminate the reaction. The mixture was allowed to stand for 15 min at 4°C to precipitate unhydrolyzed protein substrate, followed by centrifuging at 7,000 x *g* for 10 min. The supernatant was determined for protein content by the Lowry assay (Lowry *et al*., 1951) using tyrosine as a standard. One unit of activity was defined as that releasing 1 µmole of tyrosine per min. A blank was prepared in the same manner, except PCE was added after addition of 50% TCA.

2.3.4.2 Amidase activity assay

Amidase activity of PCE was measured using BAPNA as a substrate as described by Hau and Benjakul (2006). Sample (200 µL) was added with 200 µL of distilled water and 1000 µL of reaction buffer. The reaction was initiated by adding 200 µL of BAPNA (2 mg/mL) to the reaction mixture and mixed thoroughly. After incubation for 10 min under assay condition, 200 µL of 30% acetic acid (v/v) was added to terminate the reaction. Production of *p*-nitroaniline was measured by monitoring the absorbance of reaction mixture at 410 nm. Blank was conducted in the same manner except that PCE or fraction was added after addition of 30% acetic acid. Trypsin amidase activity was calculated using the following equation:

$$\text{Amidase activity (µmol/min/mL)} = \frac{(A - A_0) \times \text{mixture volume (mL)} \times 1{,}000}{8{,}800 \times \text{reaction time (min)} \times 0.2}$$

where 8,800 ($cm^{-1}M^{-1}$) is the molar extinction coefficient of *p*-nitroaniline; A and A_0 are A_{410} of sample and blank, respectively.

2.3.4.3 Esterase activity assay

Esterase activity of PCE was determined by the method of Klomklao *et al*. (2007b) using TAME as substrate. Sample (20 µL) was mixed with 3.0 mL of 1 mM TAME in different reaction buffers and incubated at the selected temperature for 20 min. Production of *p*-tosyl-arginine was measured by monitoring the increase in absorbance at 247 nm. One unit of esterase activity was defined as the amount causing an increase of 1.0 in absorbance at 247 nm per minute.

2.3.5 Temperature and pH profile

The temperature profile of PCE was studied at various temperatures (25, 35, 45, 50, 55, 60, 65 and 70°C) at pH 8.0. Proteolytic activity, amidase activity and esterase activity of PCE were determined in the presence of 10 mM $CaCl_2$ using casein, BAPNA and TAME as substrates, respectively. For the pH profile, the activities were studied over the pH range of 4-11, using the different buffers: 50 mM acetic acid-sodium acetate for pHs 4-6; 50 mM Tris-HCl buffer for pHs 7-9 and 50 mM glycine-NaOH buffer for pHs 10-11. The optimal temperature was used for the assay.

2.3.6 Effect of protease inhibitor

Effects of different protease inhibitors toward the proteolytic activity of PCE were determined as described by the method of Klomklao *et al*. (2007b). PCE was incubated with an equal volume of protease inhibitor solutions to obtain the final concentrations designated (0.1 mM E-64, 1 mM iodoacetic acid, 1.0 g/L SBTI, 5 mM TLCK, 5 mM TPCK, 0.01 mM pepstatin A and 2 mM EDTA). The mixture was incubated at room temperature (25-28°C) for 15 min. The remaining proteolytic activity, amidase activity and esterase activity were determined. The percent inhibition was then calculated. The control was conducted in the same manner except that deionized water was used instead of inhibitors.

2.3.7 Preparation of PCE ammonium sulfate fraction

PCE was subjected to ammonium sulfate precipitation at 40-60% saturation according to the method of Hau and Benjakul (2006). The mixture was centrifuged at 11,200 x *g* for 30 min at 4°C and the pellet obtained was re-dissolved in 50 mM Tris-HCl buffer, pH 8.0. The dissolved pellet was dialyzed against 15 volumes of the same buffer overnight at 4°C with three changes of dialysis buffer. The dialysate was kept at 4°C not longer than 2 days before use and defined as ammonium sulfate fraction (ASF). ASF was determined for proteolytic activity using different substrates under optimal condition.

2.3.8 Activity staining

ASF from pyloric caeca of different fish species was separated on SDS-PAGE (Laemmli, 1970), followed by activity staining according to the method of Klomklao *et al*. (2004). ASF was mixed with sample buffer (0.125 M Tris-HCl, pH 6.8 containing 4% SDS and 20% (v/v) glycerol) at a ratio of 1:1 (v/v). Six µg of proteins were loaded onto the gel made of 4% stacking and 12% separating gels. 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). After electrophoresis, gels were immersed in 100 mL of 50 mM Tris-HCl buffer, pH 7.5 containing 2% casein (w/v) for 1 h with constant agitation at 0°C to allow the substrate to penetrate into the gels. Thereafter, the gels were transferred to 2% casein (w/v) in 50 mM Tris-HCl buffer with optimal pH of each fish species, followed by incubation at optimal temperature for 15 min with constant agitation. The gels were then fixed and stained with 0.125% Coomassie Blue R-250 in 50% methanol and 10% acetic acid and destained in 30% methanol and 10% acetic acid. Development of clear zones on blue background indicated proteolytic activity.

For the inhibitor study, ASF was incubated with an equal volume of inhibitor solutions to obtain the final concentration designated (1.0 and 2.0 g/L soybean trypsin inhibitor and 5 and 10 mM TLCK) for 15 min at room temperature.

After incubation, the mixtures were mixed with sample buffer at a ratio of 1:1 (v/v). The mixtures were loaded onto the gel and activity staining was performed as previously described.

2.3.9 Preparation of gelatin hydrolysate from fish skin

2.3.9.1 Preparation of gelatin from fish skin

Fish skin gelatin was extracted according to the method of Jongjareonrak *et al*. (2006). Skin of brownstripe red snapper was cut into small pieces (0.5 x 0.5 cm). Treated skin was mixed with 10% butyl alcohol with a solid/solvent ratio of 1:10 (w/v) for 18 h and the solvent was changed every 6 h. Defatted skin was washed with 10 volumes of cold water for three times. Defatted skin was gently stirred with 10 volumes of 0.2 M NaOH at 4°C. The solution was changed every 30 min for 3 times to remove non-collagenous proteins and pigments. Alkaline treated skin was drained and rinsed with tap water until neutral pH was obtained. The skin was then swollen with 10 volumes of 0.05 M acetic acid at room temperature for 3 h with a gentle stirring. Swollen skin was drained and rinsed with tap water until neutral pH was obtained. A neutral swollen skin was extracted with 10 volumes of distilled water at 45°C for 12 h with continuous stirring. The mixture was then filtered in a Buchner funnel with a Whatman No. 4 filter paper. The resulting filtrate was freeze-dried to obtain gelatin powder.

2.3.9.2 Effect of ASF concentrations and hydrolysis time on DH

Gelatin powder was mixed with 50 mM Tris-HCl buffer with pH showing maximal activity for each species to obtain the concentration of 2% (w/v). Gelatin solutions were incubated at optimal temperature for proteolytic activity of each species for 10 min. ASF with amidase activity of 1 and 2 unit/g gelatin was added into the mixtures. 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 at 90°C for 10 min. 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 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.

2.3.9.3 Preparation of gelatin hydrolysate with different DHs

After pH adjustment and incubating at optimal temperature for 10 min, gelatin solutions were added with ASF to obtain different amidase activity (1, 2, 3, 4, and 5 unit/g gelatin) and mixed thoroughly. After 60 min of hydrolysis, 1 mL of sample was mixed with 1 mL of 1% SDS solution (90°C) and placed in a water bath at 90°C for 10 min. DH of gelatin hydrolysate was determined as described by Benjakul and Morrissey (1997). Log_{10} of activity unit was plotted against % DH to obtain the regressive equation. From the relationship, the amount of enzyme required for hydrolysis to obtain DHs of 5, 10 and 15% was calculated and used for gelatin hydrolysate preparation. The hydrolysates were clarified by centrifuging at 2,000 x *g* for 5 min at 4°C to remove insoluble debris. The supernatant was collected and referred to as fish skin gelatin hydrolysate. Hydrolysates were subjected to analyses of protein pattern and antioxidative activities.

2.3.10 Protein pattern of fish skin gelatin hydrolysate

Protein patters of fish skin gelatin hydrolysates were determined using SDS-PAGE (Laemmli, 1970) with 4.5% stacking gel and 20% separating gel

according to method of Klompong *et al*. (2008). Hydrolysates 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 1:1 (v/v). Proteins (15 μg) were loaded onto the gel. The electrophoresis was run at a constant current of 15 mA per gel by a Mini-Protean II Cell apparatus. The gels were fixed and stained with 0.05% (w/v) Coomassie Brilliant Blue R-250 in 15% methanol and 5% acetic acid and destained in 30% methanol and 10% acetic acid. Wide range molecular weight marker was used to estimate the molecular weight of hydrolysates.

2.3.11 Determination of antioxidative activities

2.3.11.1 DPPH radical scavenging activity

DPPH radical scavenging activity of gelatin hydrolysates was determined as described by Binsan *et al*. (2008) with a slight modification. Sample solution (1.5 mL) was added with 1.5 mL of DPPH in 95% ethanol. The mixture was mixed vigorously and allowed to stand for 30 min in dark at room temperature. The absorbance of resulting solution was measured at 517 nm. The activity was expressed as μmol Trolox equivalents (TE)/g protein. Distilled water was used instead of the sample and prepared in the same manner to obtain the control.

2.3.11.2 ABTS radical scavenging activity

ABTS radical scavenging activity was determined according to the method of Re *et al*. (1999) with a slight modification. ABTS was dissolved in water to obtain a concentration of 7.4 mM and used as ABTS stock solution. ABTS radical ($ABTS^{\bullet+}$) was produced by reacting ABTS stock solution with 2.6 mM potassium persulfate at the ratio of 1:1 (v/v) and allowing the mixture to react in dark at room temperature for 12 h before use. The $ABTS^{\bullet+}$ solution was diluted with methanol to obtain an absorbance of 1.1 (±0.02) at 734 nm. To initiate the reaction, 150 μL of sample was mixed with 2.85 mL of diluted ABTS•+ solution. The extent of quenching of the $ABTS^{\bullet+}$ was measured at 734 nm after 2 h incubation at room temperature in

dark. The control was prepared in the same manner except that distilled water was used instead of the sample. The activity was expressed as μmol TE/g protein.

2.3.11.3 Ferric reducing antioxidant power (FRAP) assay

The capacity of gelatin hydrolysates to reduce ferric-tripyridyltriazine complex were evaluated by FRAP assay as described by Benzie and Strain (1996) with a slight modification. A 2.85 mL of freshly prepared FRAP reagent (2.5 mL of a 10 mM TPTZ solution in 40 mM HCl plus 2.5 mL of 20 mM $FeCl_3.6H_2O$ solution and 25 mL of 300 mM acetate buffer, pH 3.6) was incubated at 37°C for 30 min before mixing with 150 μL of sample. The mixture was allowed to react in dark at room temperature. Absorbance at 593 nm was read after 30 min of reaction. The control was prepared in the same manner except that distilled water was used instead of the sample. FRAP was expressed as μmol TE/g protein.

2.3.12 Protein determination

Protein concentration was measured by the method of Lowry *et al*. (1951) using bovine serum albumin as a standard.

2.3.13 Statistical analysis

Experiments were run in triplicate. All data were subjected to analysis of variance (ANOVA) and differences between means were evaluated by Duncan's Multiple Range Test (Steel and Torrie, 1980). SPSS Statistic Program (Version 10.0) (SPSS Inc, Chicago, IL, USA) was used for data analysis.

2.4 Results and Discussion

2.4.1 Temperature and pH profile of proteases in PCE

The effect of temperatures on proteolytic activity of PCE from different fish species using various substrates is depicted in Figure 4. PCE from bigeye snapper and threadfin bream showed the highest hydrolytic activities at 60 and 55°C, respectively, when all substrates including casein, BAPNA and TAME were used as substrates. For PCE from brownstripe red snapper, the optimal temperature was 65^{o}C when casein was used as substrate and the maximal activity was found at 60^{o}C when BAPNA and TAME as substrates were used. The results suggested that all PCE had the maximal activity at high temperatures ranging from 55 to 65^{o}C. Activities of all PCE decreased markedly when temperature was above 65^{o}C, mainly due to the thermal denaturation (Klomklao *et al*., 2007d). Additionally, it was found that optimal temperature of proteases in PCE could be governed by substrates used.

The pH profile of PCE from different fish species is shown in Figure 5. For all substrates used, the highest activity was obtained at pH 8.0 for PCE from brownstripe red snapper and bigeye snapper. Nevertheless, PCE from threadfin bream exhibited the highest activity at pH 8.5. Thus, alkaline proteases were dominant in PCE of all species tested. Optimal temperature and pH of proteases extracted from internal organ of fish species vary with organs, species, etc. Klomklao *et al*. (2004) reported that trypsin from splenic extracts from skipjack tuna (*Katsuwonus pelamis*), yellowfin tuna (*Thunnus albacores*) and tongol tuna (*Thunnus tonggol*) showed the maximal activity at the same pH and temperature (9.0 and 55°C) when casein was used as a substrate. Purified trypsin from pyloric caeca of Monterey sardine (*Sardinops sagax caerulea*) had optimal pH and temperature of pH 8.0 and 50°C, respectively, for both esterase activity toward TAME and amidase activity against BAPNA (Castillo-Yañez *et al*., 2005). Trypsin from pyloric caeca of chinook salmon (*Oncorhynchus tshawytscha*) had the optimal pH and temperature at 8.0 and 60°C using BAPNA as substrate (Kurtovic *et al*., 2006). Siringan *et al*. (2007) reported that optimal temperature and pH of trypsin-like proteinases from whole Indian anchovy

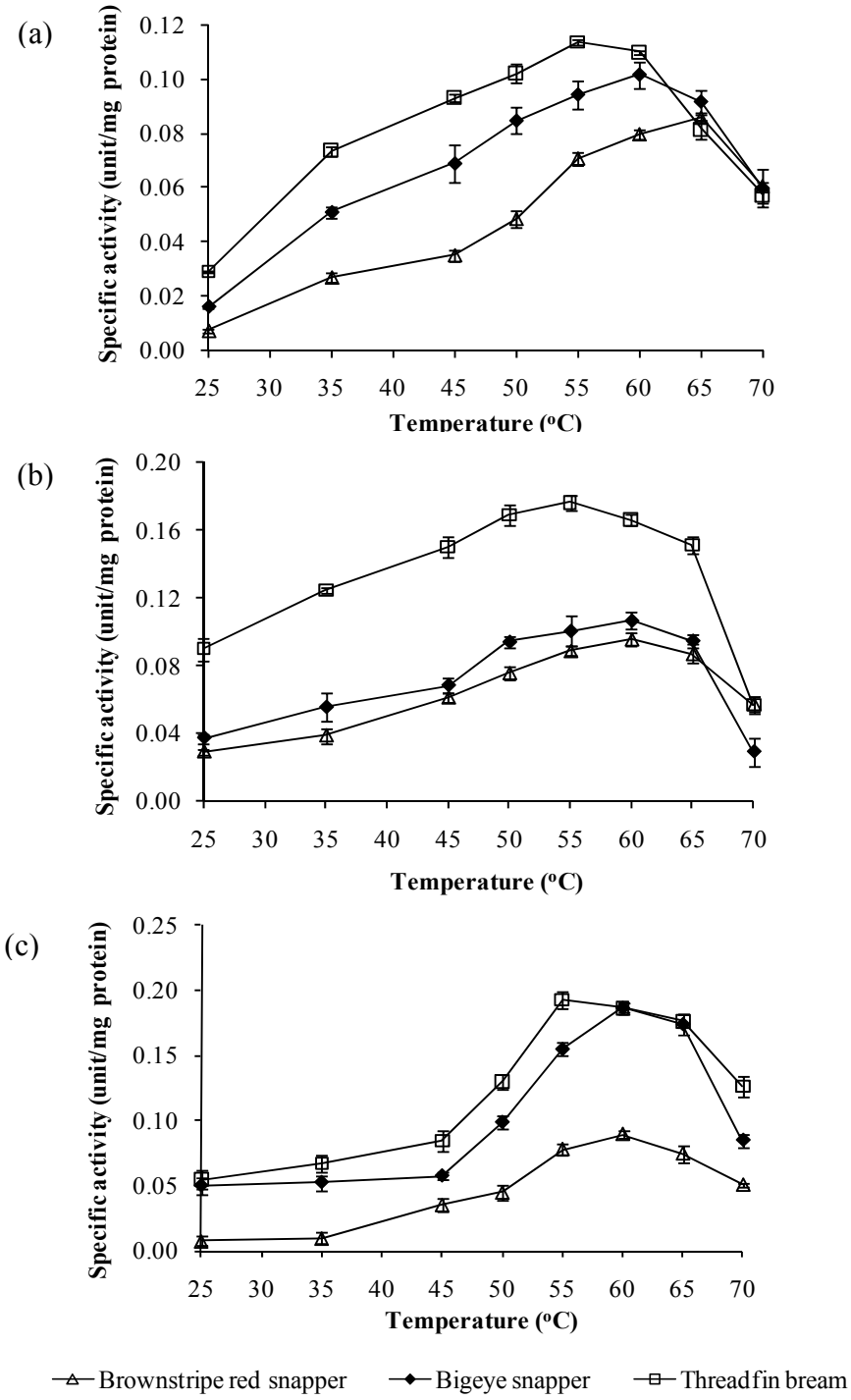

Figure 4. Temperature profile of proteases in pyloric caeca extract from different fish species using casein (a), BAPNA (b) and TAME (c) as substrates. The reactions were performed at pH 8.0. Bars represent the standard deviation (n=3).

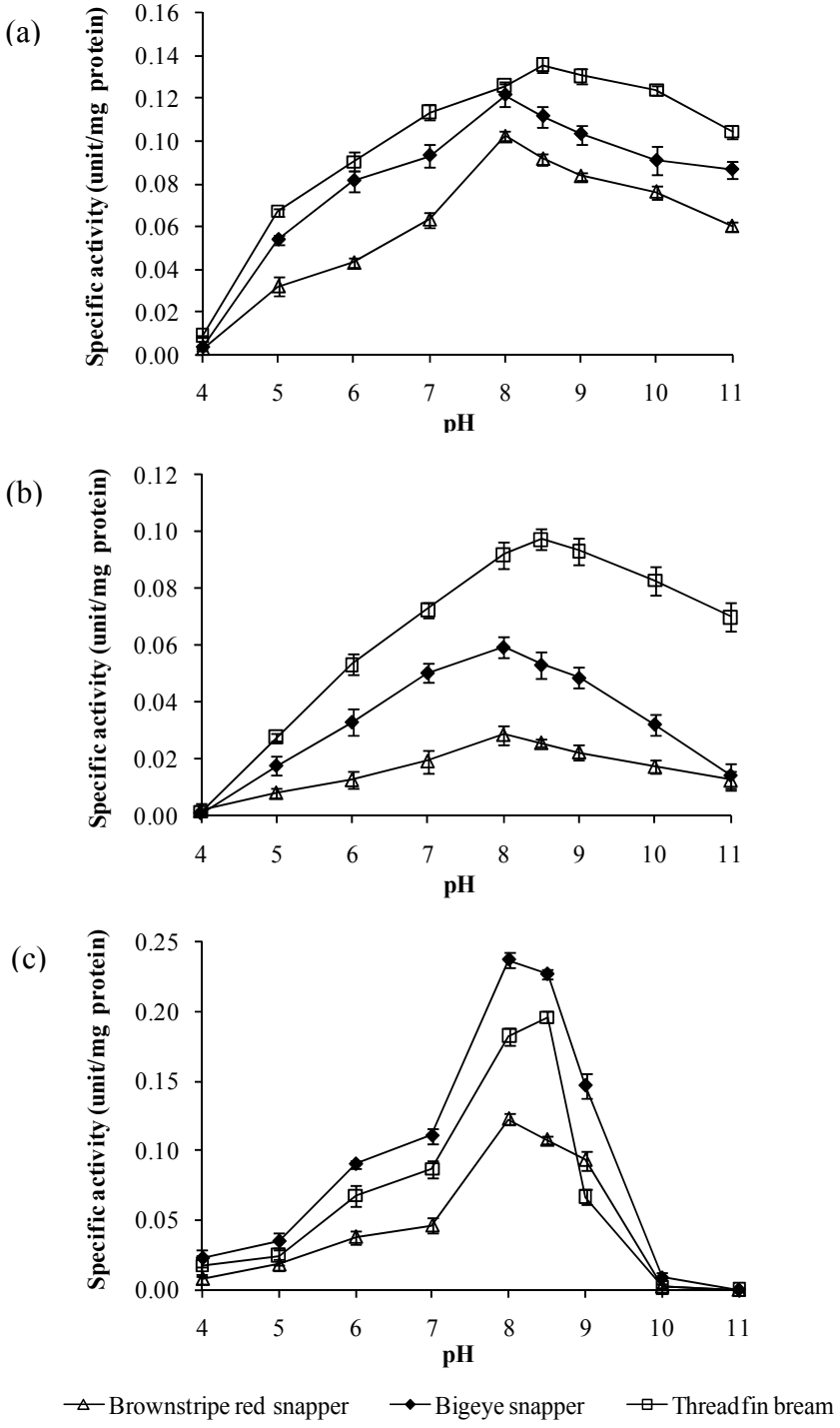

Figure 5 pH profile of proteases in pyloric caeca extract from different fish species using casein (a), BAPNA (b) and TAME (c) as substrates. The reactions were performed at 55°C and 60°C with all substrates for threadfin bream and bigeye snapper, respectively and at 60°C with BAPNA and TAME and 65°C with casein for brownstripe red snapper. Bars represent the standard deviation (n=3).

(*Stolephorus* spp.) were 50-60°C and 8.5, respectively when Boc-Asp(oBzl)-Pro-Arg-AMC was used as substrate. Shi *et al*. (2007) found that the optimal activity of purified trypsin from pyloric caeca of New Zealand hoki fish (*Macruronus Novaezealandlae*) for the hydrolysis of BAPNA was at pH 9.0 and 60°C. Furthermore, the optimal pH and temperature of trypsin from pyloric caeca of bluefish (*Pomatomus saltatrix*) for the hydrolysis of BAPNA were 9.5 and 55°C, respectively (Klomklao *et al*., 2007d). In general, trypsins tend to be more active at alkaline pH, with pH optimum ranging from 7.5 to 10.5 and temperature between 40 and 60°C (Klomklao *et al*., 2006b).

Under the optimal temperature and pH, PCE from threadfin bream exhibited the highest specific activity when casein and BAPNA were used as substrates. However, PCE from bigeye snapper showed the higher specific activity when TAME was used. Based on the same protein content of extract, PCE from threadfin bream therefore showed the higher amidase activity but lower esterase activity than PCE from bigeye snapper. Among all PCEs tested, that from brownstripe red snapper had the lowest specific activity.

2.4.2 Effect of protease inhibitors on proteolytic activity of PCE

The impact of various inhibitors on proteolytic activity of PCE from different species is shown in Table 5. For all PCE, the activities determined using different substrates were strongly inhibited by TLCK (trypsin specific inhibitor). The inhibition was more pronounced (96-99% inhibition) when BAPNA and TAME, the specific substrates for trypsin, were used. SBTI, the serine proteinase inhibitor, was also exhibited the effective inhibition on proteases in PCE (75-99% inhibition). The activity was partially inhibited by TPCK (2-40%), the specific inhibitor for chymotrypsin, especially when casein was used as substrate. Chymotrypsin was also found in pyloric caeca of marine fish (Castillo-Yañez *et al*., 2005). Therefore, PCE might contain chymotrypsin at some levels. Cysteine proteinase inhibitors including E-64 and iodoacetic acid slightly inhibited proteinase activity (1-6%). Additionally, EDTA, a metalloproteinase inhibitor and pepstatin A, an aspartic proteinase inhibitor,

Table 5. Effects of various inhibitors on % inhibition of proteolytic activities of pyloric caeca extract from different fish species.

Substrates	Inhibitors		Pyloric caeca extract		
	Types	Final concentrations	Brownstripe red snapper[1]	Bigeye snapper[2]	Threadfin bream[3]
casein	E-64	0.1 mM	1	3	3
	EDTA	2 mM	4	1	1
	Iodoacetic acid	1 mM	1	4	1
	SBTI	1.0 g/L	90	87	90
	TLCK	5 mM	83	74	88
	TPCK	5 mM	40	36	30
	Pepstatin A	0.01 mM	7	8	4
BAPNA	E-64	0.1 mM	1	1	2
	EDTA	2 mM	2	3	1
	Iodoacetic acid	1 mM	1	3	1
	SBTI	1.0 g/L	88	98	99
	TLCK	5 mM	99	99	99
	TPCK	5 mM	16	11	24
	Pepstatin A	0.01 mM	1	0	0
TAME	E-64	0.1 mM	5	7	6
	EDTA	2 mM	5	6	8
	Iodoacetic acid	1 mM	3	4	4
	SBTI	1.0 g/L	75	76	86
	TLCK	5 mM	96	96	97
	TPCK	5 mM	2	10	4
	Pepstatin A	0.01 mM	2	3	1

[1] Activities were assayed at pH 8.0 and 65°C when casein was used as substrate, and 60°C when BAPNA and TAME were used as substrates
[2] Activities were assayed at 60°C and pH 8.0 for all substrates used
[3] Activities were assayed at 55°C and pH 8.5 for all substrates used

had low inhibitory effect (0-8% inhibition) on proteolytic activity of PCE for all substrates used. Klomklao *et al*. (2004) reported that proteases from spleen extracts of skipjack tuna, yellowfin tuna and tongol tuna were identified to be trypsin or trypsin-like enzymes based on the inhibitory study. Siringan *et al*. (2007) indicated that the proteinases from whole Indian anchovy were trypsin-like proteinases, which were inhibited by SBTI and TLCK. The enzymes from pyloric caeca of walleye pollack were most likely trypsin due to the strong inhibition by SBTI and TLCK (Kishimura

et al., 2008). Based on inhibition study, major proteases of PCE belonged to serine proteases, more likely trypsin-like enzymes.

2.4.3. Fractionation of PCE using ammonium sulfate precipitation

Specific activity, purity and yield of ammonium sulfate fraction (ASF) of PCE from different species are shown in Table 6. Specific proteolytic activities of all ASFs were higher than those of PCE for all substrates tested. Specific activities of all samples using the ester substrate (TAME) were higher than that on the amide substrate (BAPNA), indicating the preference of enzyme in hydrolyzing TAME. After fractionation, purity and yield of ASF increased by 3.6-7.8 fold and 47-77 %, compared to those obtained in PCE.

Hau and Benjakul (2006) reported that after 40-60% saturated ammonium sulfate precipitation of crude extract of pyloric caeca from bigeye snapper (*Priacanthus macracanthus*), a purification fold of 4.5 was obtained with the yield of 69%. When trypsin from the pyloric caeca of bluefish was precipitated with ammonium sulfate at 40-60% saturation, specific activity was increased by 8.3-fold with the yield of 60% (Klomklao *et al*., 2007d). Precipitation of all PCE with 40-60% saturated ammonium sulfate increased the specific activity and purity, suggesting the removal of some contaminating proteins from the extract (Klomklao *et al*., 2007d).

2.4.4 Activity staining of ASF from PCE

From activity staining, the activity bands of ASF from PCE of brownstripe red snapper were observed with apparent molecular weight (MW) of 20, 24-29, 45 and 97 kDa (Figure 6(a)). The MW of 17, 20, 22, 45 and 97 kDa were estimated from the activity bands of ASF of bigeye snapper PCE (Figure 6(b)) and threadfin bream ASF showed activity bands at 20, 22, 36 and 45 kDa (Figure 6(c)). MW of trypsin was generally 24 kDa (Torrissen and Male, 2000). MW of trypsin-like enzymes from hepatopancreas of crawfish (*Procambarus clarkia*) were between 35 and 41 kDa (Jeong *et al*., 2000). Trypsin-like enzyme isolated from pyloric caeca of

Table 6. Fractionation of pyloric caeca extract of different fish species using 40-60% saturated ammonium sulfate precipitation.

Substrates	Parameters	Brownstripe red snapper		Bigeye snapper		Threadfin bream	
		PCE	ASF	PCE	ASF	PCE	ASF
casein[1]	Total activity (unit)	47.21	32.29	38.52	29.68	84.59	40.62
	Specific activity (unit/mg protein)	0.35	1.82	0.30	2.35	0.58	2.11
	Yield (%)	100	68	100	77	100	48
	Purity (fold)	1.0	5.2	1.0	7.8	1.0	3.6
BAPNA[2]	Total activity (unit)	15.97	11.15	16.63	12.79	40.98	25.41
	Specific activity (unit/mg protein)	0.12	0.63	0.14	1.01	0.29	1.32
	Yield (%)	100	70	100	77	100	62
	Purity (fold)	1.0	5.1	1.0	7.3	1.0	4.6
TAME[3]	Total activity (unit)	13.84	7.40	12.69	6.55	24.46	11.51
	Specific activity (unit/mg protein)	0.22	1.18	0.20	1.23	0.35	2.13
	Yield (%)	100	53	100	52	100	47
	Purity (fold)	1.0	5.4	1.0	6.1	1.0	6.1

[1]one unit was defined as that releasing 1 µmole of tyrosine per min
[2]one unit was defined as that releasing 1 µmole of *p*-nitroaniline per min
[3]one unit was defined as that causing an increase of 1.0 in absorbance at 247 nm per min
PCE: pyloric caeca extract
ASF: ammonium sulfate (40-60% saturation) fraction

tambaqui (*Colossoma macropomum*) had MW of 38.5 kDa (Bezerra *et al*., 2001). The results indicated the differences in the proteases in pyloric caeca among all fish species tested in terms of type, isoform and amount.

SBTI at both levels of 1 and 2 g/L SBTI was able to completely inhibit the activity bands of PCE with MW of 20 kDa for brownstripe red snapper, 20 kDa for bigeye snapper and 20, 22 and 36 kDa for threadfin bream. For activity bands with MW of 24 kDa of brownstripe red snapper and 22 kDa of bigeye snapper, 2.0 g/L SBTI showed higher inhibitory activity than 1.0 g/L SBTI as evidenced by less intensity of activity band. Inhibition of some proteases was mostly dependent upon the concentration of SBTI. The inhibitions on activity bands by TLCK (5 and 10 mM)

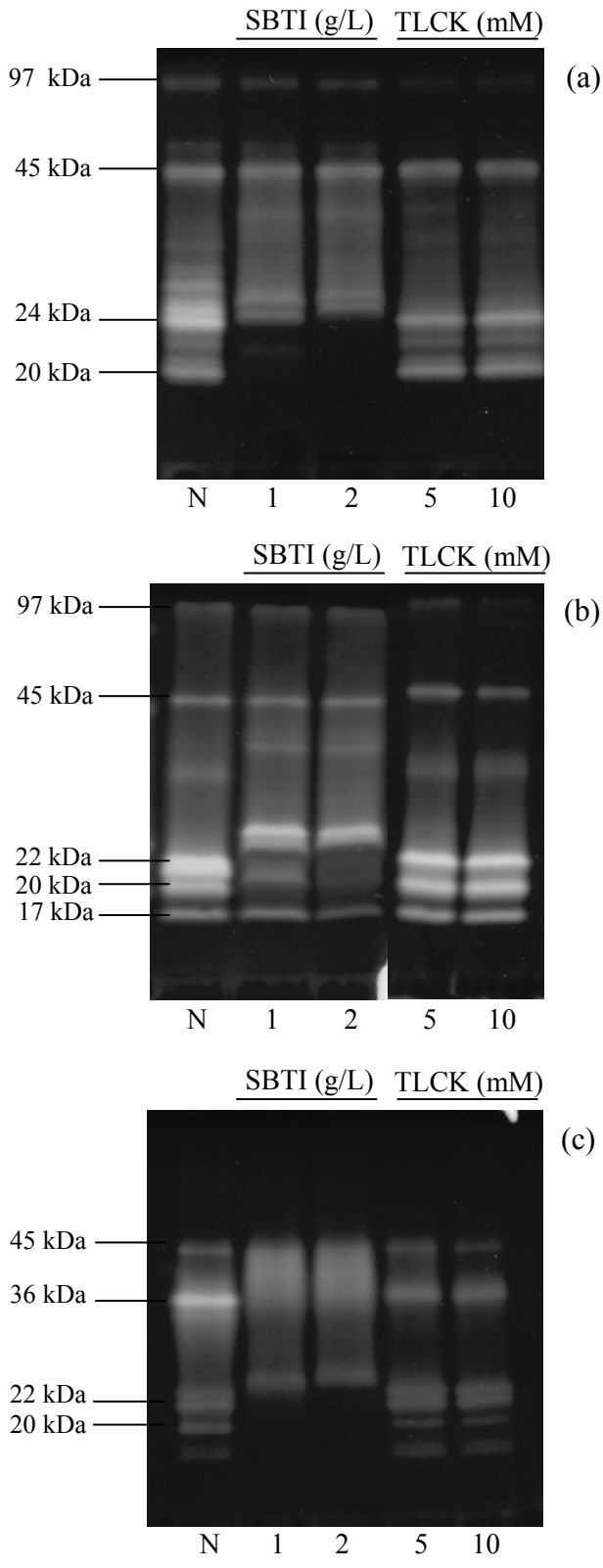

Figure 6. Activity staining toward casein of ASF from pyloric caeca of brownstripe red snapper (a), bigeye snapper (b) and threadfin bream (c). N: no inhibition. Numbers designate the concentration of inhibitors used.

were different from those obtained using SBTI. Only the activity bands with MW of 24 kDa from brownstripe red snapper, 22 kDa of bigeye snapper and 20 and 36 kDa of threadfin bream were partially inhibited as indicated by the lowered band intensity. No differences in activity zone were found between TLCK at 5 and 10 mM. The differences in inhibition of inhibitors on activity bands were possibly associated with the different specificity in inhibition of target enzymes. Activity bands including those with MW of 45 and 97 kDa remaining after the treatment with SBTI and TLCK most likely represented other proteases, especially non-serine proteases. Based on the MW and inhibition study, pyloric caeca of all species contained trypsin-like enzyme with MW of 24 kDa for brownstripe red snapper, 22 kDa for bigeye snapper and 20 or 36 kDa for threadfin bream.

2.4.5 Hydrolysis of fish skin gelatin using ASF of PCE

Degree of hydrolysis (DH) is essential because several properties of protein hydrolysates are closely related to DH (Adler-Nissen, 1979; Nielsen, 1997). Brownstripe red snapper skin gelatin hydrolyzed using ASF from PCE of different species showed an initial rapid hydrolysis rate followed by a slower hydrolysis rate (Figure 7). 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, 2000b).

Rapid hydrolysis was observed within the first 40 min when ASF of brownstripe red snapper was used, whereas ASF from bigeye snapper and threadfin bream exhibited the rapid rate of hydrolysis within the first 20 min. At the same hydrolysis time, higher DH was observed with higher activity unit of ASF. The results indicated that the higher amount of proteases in ASF, the more peptide bonds were cleaved (Klompong *et al*., 2008).

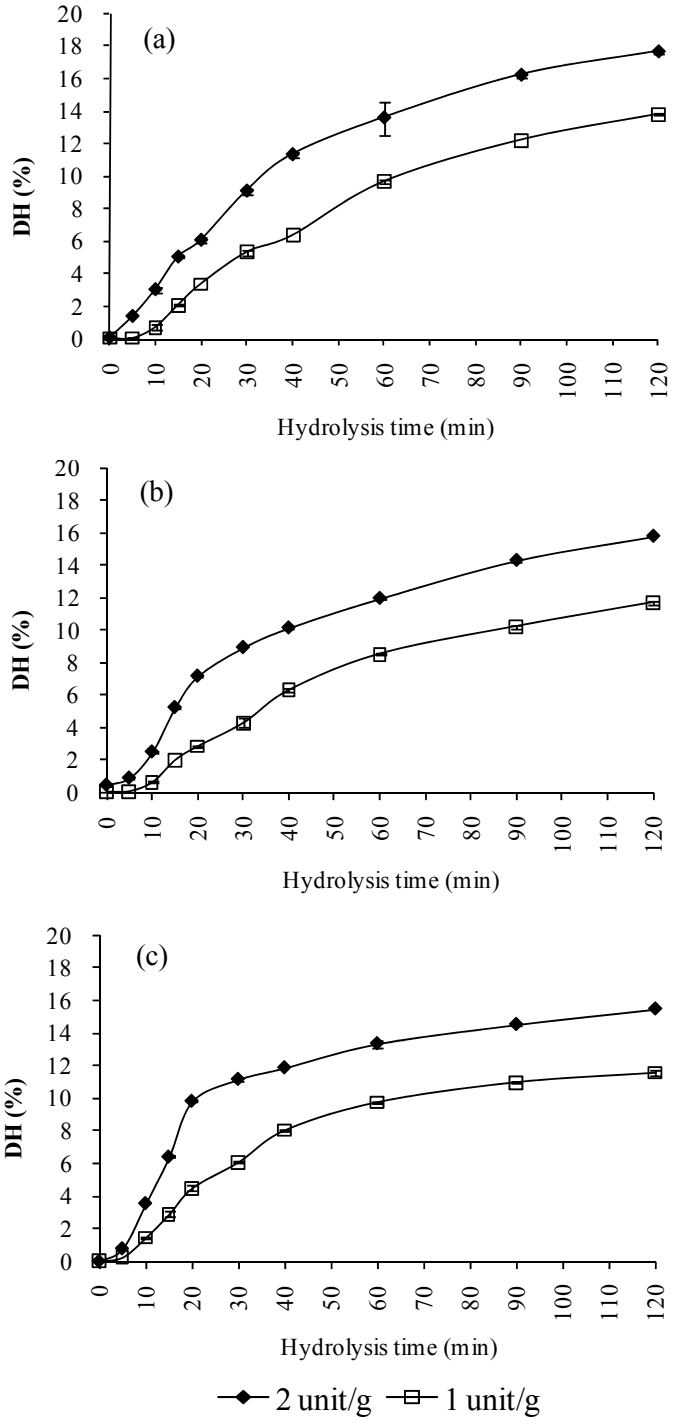

Figure 7. Changes in degree of hydrolysis (DH) of brownstripe red snapper skin during hydrolysis with ASF of pyloric caeca from brownstripe red snapper (a), bigeye snapper (b) and threadfin bream (c). The reactions were performed at pH 8.0, 60^{o}C for brownstripe red snapper and bigeye snapper and pH 8.5, 55^{o}C for threadfin bream.

When $\log_{10}$ (unit activity) of each ASF was plot against DH, a linear relationship and the regressive equations were obtained (Figure 8). Benjakul and Morrissey (1997) found a similar linear relationship between %DH and log of enzyme concentration, when hydrolyzing Pacific whiting (*Merluccius productus*) solid waste with Alcalase and Neutrase. This relationship is further supported by Gbogouri *et al*. (2004) for salmon head protein hydrolysates derived using Alcalase and Klompong *et al*. (2008) for yellow stripe trevally derived with Alcalase and Flavourzyme. At the same unit of proteolytic activity, skin gelatin treated with brownstripe red snapper ASF (HBR) showed higher DH than that treated with bigeye snapper ASF (HB) and threadfin bream ASF (HT), indicating a higher hydrolytic activity of brownstripe red snapper proteases towards fish skin gelatin, compared with proteases from other species. From the regressions, amount of ASF required to prepare fish skin gelatin hydrolysate with DHs of 5, 10 and 15% could be calculated.

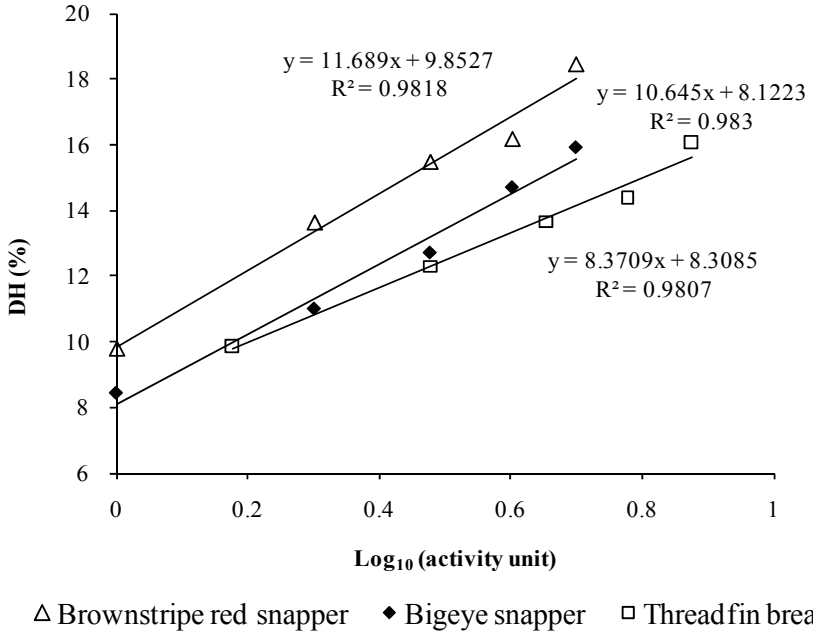

Figure 8. Relationship between degree of hydrolysis (DH) of skin gelatin and $\log_{10}$ (unit of amidase activity) of ASF from different fish species. The reaction was performed for 60 min at pH 8.0, 60°C for brownstripe red snapper and bigeye snapper and pH 8.5, 55°C for threadfin bream.

2.4.6 Protein pattern of skin gelatin hydrolysate

Protein patterns of gelatin from brownstripe red snapper skin and gelatin hydrolysates prepared using ASF of brownstripe red snapper (HBR), bigeye snapper (HB) and threadfin bream (HT) with 5, 10 and 15% DHs are shown in Figure 9(A) and (B), respectively. No differences in protein pattern of skin gelatin under reducing and non-reducing condition, indicating that skin gelatin contained no disulfide bond. Major constituents of fish skin gelatin were β-chain (200 kDa) and α1 and α2 (approximately 100-120 kDa).

When fish skin gelatin was hydrolyzed to different DHs, the lower molecular weight (MW) proteins or peptides were obtained with increasing DHs. No β-chain and α-chain were remained in hydrolysates with all DHs. At 5% DH, hydrolysates contained proteins with MW of 15.6 and 17.8 kDa as the major constituents. When DHs increased to 10-15%, protein bands in all hydrolysates totally disappeared. The result suggested that proteins or peptides were too small to be detected under the analytical electrophoresis conditions (Guan *et al*., 2007).

In general, the proportion of high MW peptides in cod byproduct (Šližyte *et al*., 2005) and salmon byproducts (Gbogouri *et al*., 2004; Sathivel *et al*., 2005) decreased with increasing DHs. During hydrolysis, enzymatic breakdown of protein involves a major structural change, in which the protein is slowly hydrolyzed into smaller peptide units (Klompong *et al*., 2008). Gbogouri *et al*. (2004) hydrolyzed salmon head protein with Alcalase to obtain DH of 15% and the resulting hydrolysate had MW ranging from 4.2 kDa to 13.2 kDa. Visceral waste protein hydrolysate from Indian freshwater major carp (*Catla catla*) using a neutral protease possessing 48% DH had MW less than 8 kDa, whereas visceral waste protein had MW greater than 69 kDa (Bhaskar *et al*., 2008). The results indicated that the amount of very small peptides increased with increasing DHs.

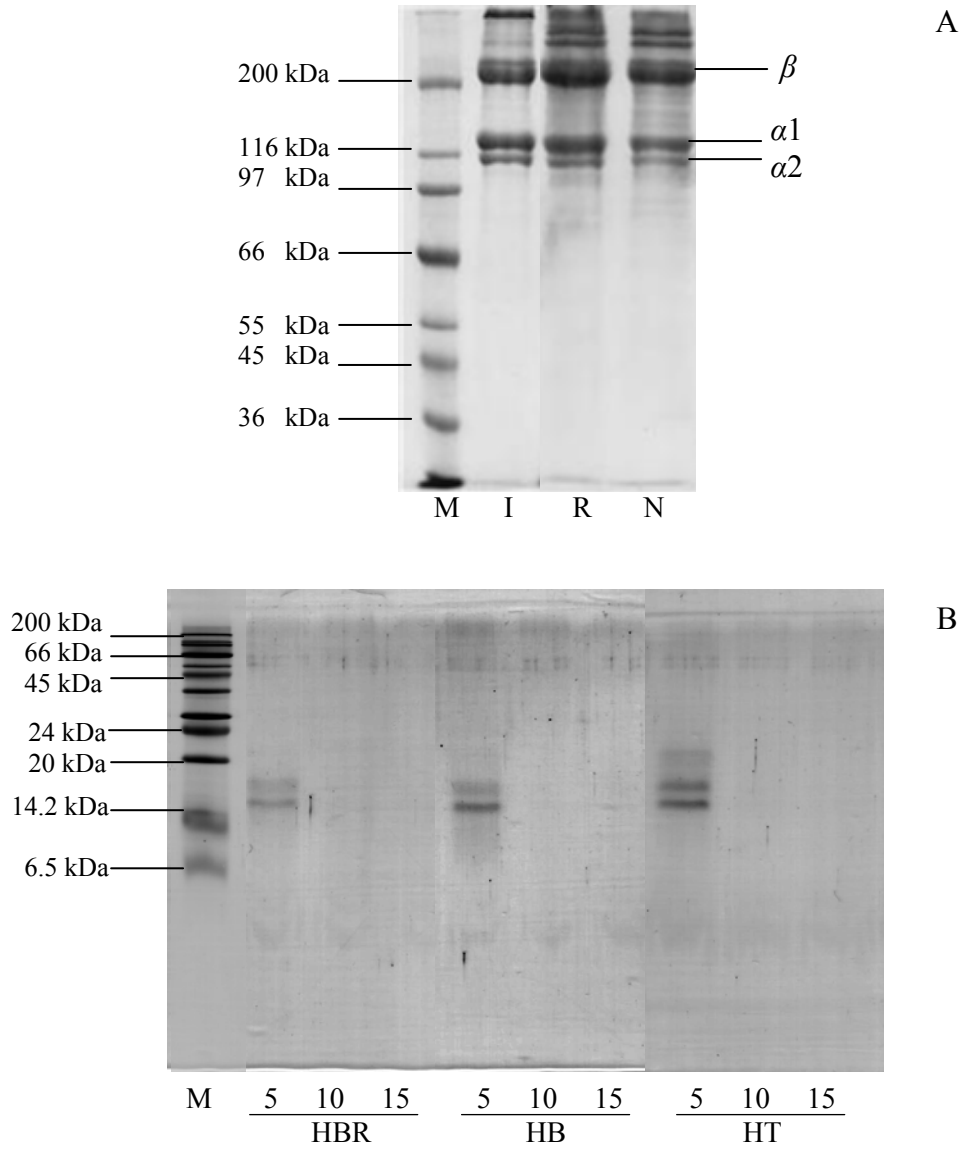

Figure 9. SDS-PAGE pattern of fish skin gelatin from brownstripe red snapper skin under reducing (R) and non-reducing (N) conditions (A). M: high molecular weight marker; I: type I collagen and SDS-PAGE pattern of gelatin hydrolysates from brownstripe red snapper skin prepared using ASF of pyloric caeca extract from brownstripe red snapper (HBR), bigeye snapper (HB) and threadfin bream (HT) with different degrees of hydrolysis (DHs) (B). M: wide range molecular weight marker. Numbers designate DH (%).

2.4.7 Antioxidative activities of gelatin hydrolysates prepared using ASF

2.4.7.1 DPPH radical scavenging activity

DPPH radical scavenging activities of HBR, HB and HT with different DHs are depicted in Figure 10(a). As the DH increased, DPPH radical scavenging activities increased ($p < 0.05$). All hydrolysates exhibited the highest activity at DH of 15%. Nevertheless, no differences were observed between HT with 5% and 10% DH ($p < 0.05$). For gelatin solution (2%) without hydrolysis, a much lower DPPH radical scavenging activity was found, compared with that obtained in the gelatin hydrolysates. DPPH is a stable free radical that shows maximal absorbance at 517 nm in ethanol. When DPPH encounters a proton-donating substance, such as an antioxidant, the radical is scavenged. The color is changed from purple to yellow and the absorbance is reduced (Shimada *et al*, 1992). The effect of antioxidants on DPPH radical scavenging was thought to be due to their hydrogen-donating ability (Binsan *et al*., 2008). DPPH radical scavenging activities were found in protein hydrolysates derived from round scad and yellow stripe trevally by Alcalase and Flavourzyme (Thiansilakul *et al*., 2007a; Klompong *et al*., 2008).

2.4.7.2 ABTS radical scavenging activity

HBR, HB and HT showed different ABTS radical scavenging activities (Figure 10(b)). The highest activity was observed in all hydrolysates with 15% DH. HBR with 5 and 10% DH showed no differences in ABTS radical scavenging activities. However, HB with 5% DH exhibited higher activity than did with 10% DH ($p < 0.05$). ABTS radical assay is an excellent tool for determining the antioxidative activity, in which the radical is quenched to form ABTS-radical complex (Binsan *et al*., 2008). At 15% DH, HBR had the highest activity, compared with other hydrolysates. The result indicated that peptides produced might be different in term of amino acid composition, sequence and chain length. Generally, all hydrolysates contained peptides or proteins which were hydrogen donors and could react with the radicals to convert them to more stable products, thereby terminating the radical chain reaction.

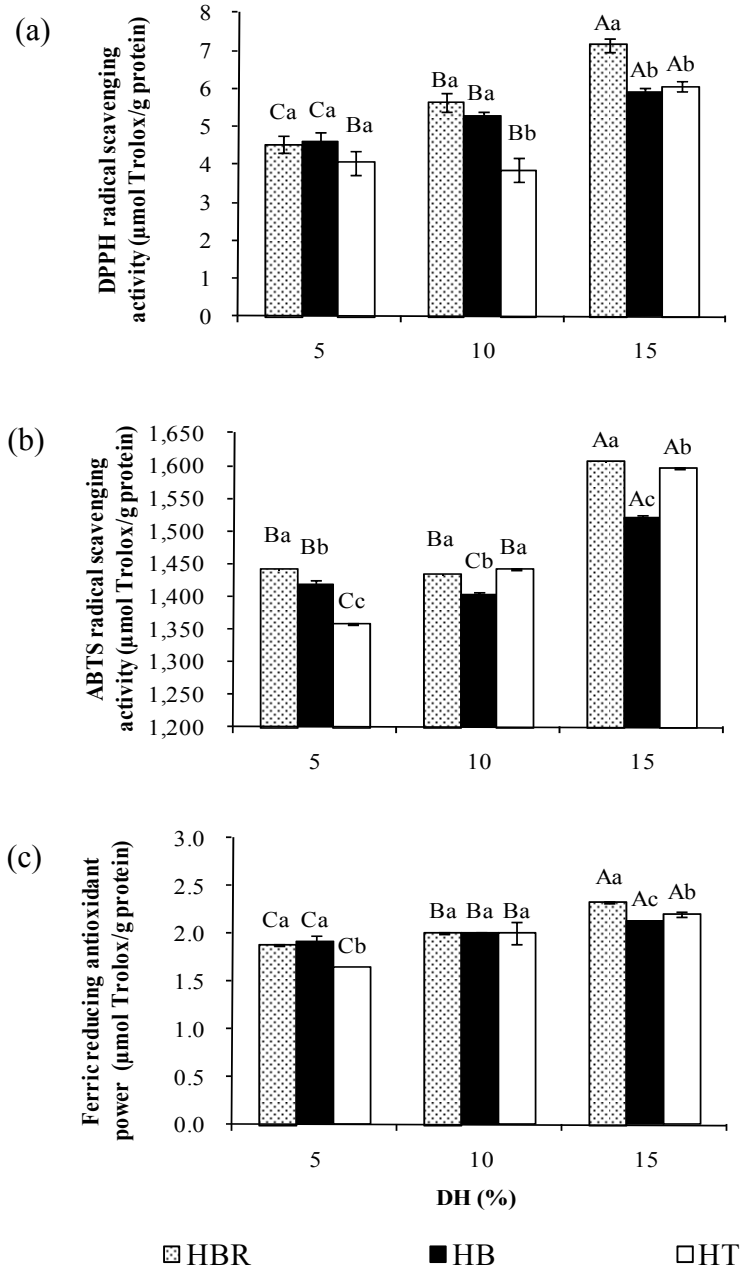

Figure 10. DPPH radical scavenging activity (a), ABTS radical scavenging activity (b) and ferric reducing antioxidant power (FRAP) (c) of gelatin hydrolysates from brownstripe red snapper skin prepared using ASF of pyloric caeca extract from brownstripe red snapper (HBR), bigeye snapper (HB) and threadfin bream (HT) with different degrees of hydrolysis (DHs). Bars represent the standard deviation (n=3). Different capital letters within the same pyloric caeca and different letters within the same degree of hydrolysis (DH) indicate significant differences ($p < 0.05$).

2.4.7.3 Ferric reducing antioxidant power

Ferric reducing antioxidant power (FRAP), generally measures the reducing ability (Binsan *et al*., 2008), of HBR, HB and HT is shown in Figure 10(c). All hydrolysates prepared using different AS fractions had the increases in FRAP when DH increased ($p < 0.05$). The result was in agreement with those of DPPH and ABTS radical scavenging activities. The greater reducing power indicated that hydrolysates could donor the electron to the free radical, leading to the prevention or retardation of propagation (Klompong *et al*., 2008). At 15% DH, HBR also showed the highest FRAP, compared with other hydrolysates ($p < 0.05$).

Therefore, the hydrolysis most likely increased the antioxidative activity of resulting hydrolysate via the enhancement of radical scavenging activity. Moreover, reducing power was increased by hydrolysis process, but the degree of increase was somewhat lower than that of radical scavenging activity.

Different antioxidative activities among HBR, HB and HT might result from existing differences in enzyme specificity toward protein substrates (Bayram *et al*., 2008). The DH greatly influenced the peptide chain length as well as the exposure of terminal amino groups of products obtained (Thiansilakul *et al*., 2007a). Klompong *et al*. (2008) also reported that the differences in antioxidative activity might be associated with the size of proteins. Wu *et al*. (2003) found that changes in size, level and composition of free amino acids of peptides also affected the antioxidative activity. Furthermore, proteases from different fish most likely cleaved the peptide bonds in fish skin gelatin at the different positions, leading to the different products with various antioxidative activities. Therefore, DH and enzyme source significantly influenced the antioxidative activity of the resulting hydrolysates. Trypsins or trypsin-like enzymes constituted as the major proteases in PCE. Thus, those enzymes could play a role in hydrolyzing the gelatin.

2.5 Conclusions

Fish pyloric caeca extracts consisted of trypsin or trypsin-like proteases with different proteolytic activity. Proteases from pyloric caeca could hydrolyze skin gelatin effectively but the resulting hydrolysate exhibited various antioxidative activity depending upon the source of pyloric caeca. Thus, hydrolysate from fish skin with enhanced antioxidative activity could be produced by the appropriate fish protease under optimal condition.

CHAPTER 3

PURIFICATION AND CHARACTERIZATION OF TRYPSIN FROM THE PYLORIC CAECA OF BROWNSTRIPE RED SNAPPER

3.1 Abstract

Trypsin was purified from the pyloric caeca of brownstripe red snapper (*Lutjanus vitta*) by ammonium sulfate (40-60% saturation) precipitation, soybean trypsin inhibitor (SBTI)-Sepharose 4B column and DEAE-Sephacel column chromatography. Purified trypsin showed a single band on sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and native-PAGE. The yield of 4.9 % with the purification fold of 20 was obtained. Trypsin had an apparent molecular weight of 23 kDa. SBTI and *N*-*ρ*-tosyl-L-lysine-chloromethylketone (TLCK) showed a strong inhibitory effect on the purified trypsin, while other protease inhibitors exhibited negligible inhibition. Trypsin had maximal activity at pH 8.5 and 60°C for the hydrolysis of α-*N*-benzoyl-DL-arginine-*ρ*-nitroanilide (BAPNA). It was stable within the temperature range of 25-55°C and pH range of 7.0-10.0. Purified trypsin had a Michaelis-Menten constant (K_m) and catalytic constant (k_{cat}) of 0.507 mM and 4.71 s^{-1}, respectively, when BAPNA was used as the substrate. For the hydrolysis of α-*N*-*ρ*-tosyl-L-arginine methyl ester (TAME), K_m and k_{cat} were 0.328 mM and 112 s^{-1}, respectively.

3.2 Introduction

Fish viscera generated during fishery processing can be used as an important source of digestive enzymes, including proteases. Proteases have been widely used for basic research and industrial applications (Shahidi and Kamil, 2001) and about 50% of the total sale of enzymes in industries consists proteases. To obtain a variety of proteases, particularly those with unique properties, the new sources of proteolytic enzymes have been studied, including proteases from fish, especially fish viscera (Souza *et al*., 2007).

Trypsin (EC 3.4.21.4) generally belongs to the group of serine proteinases. It specifically hydrolyses peptide bonds at the carboxyl terminus of lysine and arginine residues. This enzyme has been isolated from the pyloric caeca of various species of fish, including tambaqui (*Colossoma macropomum*) (Bezerra *et al*., 2001), Monterey sardine (*Sardinops sagax caerulea*) (Castillo-Yáñez *et al*., 2005), chinook salmon (*Oncorhynchus tshawytscha*) (Kurtovic *et al*., 2006), bigeye snapper (*Pricanthus macracanthus*) (Hau and Benjakul, 2006), arabesque greenling (*Pleuroprammus azonus*) (Kishimura *et al*., 2006), spotted goatfish (*Pseudupeneus maculatus*) (Souza *et al*., 2007), walleye pollock (*Theragra chalcogramma*) (Kishimura *et al*., 2008) and mandarin fish (*Siniperca chuatsi*) (Lu *et al*., 2008). Trypsin can be used for the acceleration of protein hydrolysis. Klomklao *et al*. (2006a) reported that the addition of spleen from skipjack tuna containing trypsin could enhance the protein degradation of sardine muscle during fish sauce fermentation. Additionally, trypsin was used to produce a hydrolysate with antioxidative activity (Khantaphant and Benjakul, 2008) (Chapter 2).

Brownstripe red snapper (*Lutjanus vitta*) is one of fish species used as raw materials for surimi production in Thailand (Jongjareonrak *et al*., 2006). During processing, a large amount of viscera is generated. Thus, the use of this by-product as the source of protease should maximise the utilisation of this by-product from this species. Among all viscera, pyloric caeca has been reported as the excellent source of trypsin (Hau and Benjakul, 2006). However, no information exists regarding the properties and characteristics of trypsins from pyloric caeca of brownstripe red

snapper, which can be further used as a processing aid for various industries. Therefore, this study aimed to purify and characterise trypsin from pyloric caeca of brownstripe red snapper.

3.3 Materials and Methods

3.3.1 Chemicals

α-*N*-benzoyl-DL-arginine-*p*-nitroanilide (BAPNA), *α*-*N*-*ρ*-toluene-sulfonyl-L-arginine methyl ester (TAME), soybean trypsin inhibitor (SBTI), *N*-*ρ*-tosyl-L-lysine chloromethylketone (TLCK), ethylenediaminetetraacetic acid (EDTA), iodoacetic acid, 1-(L-trans-epoxysuccinyleucylamino)-4-guanidinobutane (E-64), pepstatin A, *β*-mercaptoethanol (*β*ME), *N*-tosyl-L-phenylalanine chloromethylketone (TPCK) and wide range molecular weight protein markers were purchased from Sigma Chemical Co. (St. Louis, MO, USA). Tris (hydroxymethyl) aminomethane (Tris-HCl) was procured from Merck (Darmstadt, Germany). Diethylaminoethyl (DEAE)-Sephacel and cyanobromide (CNBr)-activated Sepharose 4B were purchased from Amersham Biosciences (Uppsala, Sweden). All chemicals were of analytical grade.

3.3.2 Preparation of pyloric caeca extract

Brownstripe red snapper were purchased from a dock in Songkhla province, Thailand. Fish were kept in ice with fish/ice ratio of 1:2 (w/w) and transported to the department of Food Technology, Prince of Songkla University within 1 h. Upon arrival, pyloric caeca from brownstripe red snapper was collected and pooled as the composite sample. Pyloric caeca was powdered in liquid nitrogen. Thereafter, the pyrolic caeca extract was prepared according to the method of Khantaphant and Benjakul (2008) (Chapter 2) with a slight modification. Pyloric caeca powder was suspended in an extraction buffer (50 mM Tris-HCl buffer, pH 8.0, containing 10 mM $CaCl_2$) with a ratio of 1:10 (w/v). The mixture was homogenised

for 2 min using a homogeniser (Model T25B, IKA labortechnik, Selangor, Malaysia) at 11,000 rpm. The homogenate was then continuously stirred for 30 min at 4°C and centrifuged at 8000 x *g* for 30 min at 4°C using a refrigerated centrifuge (Beckman Coulter Model Avant J-E, Beckman Coulter, Inc., CA, USA). The supernatant was filtered through a Whatman filter paper No. 1 (Whatman International Ltd., Maidstone, England). The filtrate obtained was refered to as 'pyloric caeca extract, PCE'.

3.3.3 Preparation of ammonium sulfate fraction

PCE was subjected to 40-60% saturation ammonium sulphate precipitation, according to the method of Khantaphant and Benjakul (2008) (Chapter 2) with a slight modification. After the addition of ammonium sulfate, the mixture was stirred gradually at 4°C for 30 min. Thereafter, the mixture was centrifuged at 8,000 x *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 of dialysis buffer. The dialysate was kept in ice and referred to as 'ammonium sulfate fraction, ASF'.

3.3.4 Purification of trypsin

ASF was purified using a series of chromatographies, including affinity column and ion-exchange column. Soybean trypsin inhibitor (SBTI)-Sepharose 4B column was prepared by 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. ASF was applied onto SBTI-Sepharose 4B column (1 x 10 cm) pre-equilibrated with 50 mM Tris-HCl, pH 7.8, containing 10 mM $CaCl_2$ and 0.5 M NaCl. The column was then 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. A fraction of 2 mL was collected and mixed rapidly with 0.5 mL of 100 mM Tris-HCl, pH 8.5 containing 10 mM $CaCl_2$. The fractions were determined for trypsin activity using BAPNA as a substrate. Active fractions were pooled and dialysed 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 was then applied onto a DEAE-Sephacel column (2.5 x 15 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 0.5 M in 10 mM Tris-HCl, pH 8.0 containing 1 mM $CaCl_2$. Fractions (2 mL) were tested for trypsin activity. Active fractions were pooled and dialysed against 10 volumes of 50 mM Tris-HCl buffer, pH 8.0 containing 10 mM $CaCl_2$ with two changes of dialysis buffer. The dialysate was lyophilised prior to the storage at -20°C.

3.3.5 Assay for trypsin activity

Trypsin activity was measured using BAPNA as a substrate according to the method of Khantaphant and Benjakul (2008) (Chapter 2). A 200 µL of sample was treated with 200 µL of distilled water and 1,000 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) was added to terminate the reaction. Production of *ρ*-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. Trypsin activity was calculated using the following equation:

$$\text{Trypsin activity (unit/mL)} = \frac{(A - A_0) \times \text{mixture volume (mL)} \times 1{,}000}{8{,}800 \times \text{reaction time (min)} \times 0.2}$$

where 8,800 ($cm^{-1}M^{-1}$) is the extinction coefficient of *ρ*-nitroaniline; A and A_0 are A_{410} of sample and blank, respectively. One unit of activity was defined as that releasing 1 nmol of *p*-nitroaniline per min.

3.3.6 Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and substrate-gel electrophoresis

SDS-PAGE was carried out according to the method of Laemmli (1970), using 12% separating gel and 4% stacking gel. Active fractions from the affinity or ion-exchange column 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 and 0.8 μg for affinity and ion-exchange fractions, respectively) 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 from the affinity fraction were stained with 0.05% (w/v) Coomassie Blue G-250 in 15% methanol and 5% acetic acid, while those from ion-exchange fraction were stained with 0.2% silver nitrate dissolved in water and 0.076% formaldehyde. Native-PAGE was performed, using 12% separating gel in a similar manner, except that the sample was not heated and all solutions contained no SDS or any reducing agent. Destaining was carried out using 30% methanol and 10% acetic acid for Coomassie Blue stained gel. Protein bands on silver stained gel were developed using 6% sodium carbonate and 0.05% formaldehyde.

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 with pH rendering the highest activity of trypsin. The mixture was incubated at 60°C for 15 min with a continueous agitation. The gels were then stained with 0.05% Coomassie Blue R-250 in 15% methanol and 5% acetic acid and destained in 30% methanol and 10% acetic acid. Proteolytic activity was indicated by the development of a clear zone on a blue background.

3.3.7 Temperature and pH profile

The temperature profile of purified trypsin was determined at various temperatures (25, 35, 45, 50, 55, 60, 65 and 70°C) at pH 8.0, using BAPNA as a substrate. For pH profile, the activity was studied over the pH range of 4.0-11.0, using the different buffers: 50 mM acetic acid-sodium acetate for pHs 4.0-6.0; 50 mM Tris-HCl buffer for pHs 7.0-9.0 and 50 mM glycine-NaOH buffer for pHs 10.0-11.0. The optimal temperature was used for the assay.

3.3.8 pH and thermal stability

The effects of pHs and temperatures on the stability of purified trypsin were evaluated by measuring the remaining trypsin activity after incubation at various pHs and temperatures. For thermal stability, purified trypsin, in 50 mM Tris-HCl containing 10 mM $CaCl_2$ (pH 8.5), was incubated at different temperatures (25, 35, 45, 50, 55, 60, 65 and 70°C) for 30 min. Then the treated samples were rapidly cooled in iced water and the residual trypsin activity was assayed as previously described.

For pH stability, purified trypsin was mixed with an equal volume of different buffers containing 10 mM $CaCl_2$ with different pHs. The mixture was allowed to stand for 30 min at room temperature (25-28°C) prior to assay at optimal pH and temperature, using BAPNA as a substrate, as described above.

3.3.9 Effect of Inhibitors

Effects of various inhibitors on the activity of purified trypsin from brownstripe red snapper were determined according to the method of Khantaphant and Benjakul (2008) (Chapter 2). Purified trypsin in 50 mM Tris-HCl buffer (pH 8.5) containing 10 mM $CaCl_2$ was added with an equal volume of protease inhibitor solutions to obtain the final concentrations designated (0.1 mM E-64, 1 mM iodoacetic acid, 1.0 g/L SBTI, 5 mM TLCK, 5 mM TPCK, 0.01 mM pepstatin A and

2 mM EDTA). The mixture was allowed to stand at room temperature (25-28°C) for 15 min. The remaining activity was determined and the percentage inhibition was then calculated. The control was conducted in the same manner except that deionised water was used instead of inhibitors

3.3.10 Kinetic studies

Kinetic studies of purified trypsin from brownstripe red snapper were carried out according to the method of Hau and Benjakul (2006) using BAPNA and TAME, ranging from 0.01 to 2.0 mM, as the substrates. The final enzyme concentration for the assay was 0.0055 mg protein/mL. The kinetic parameters including the maximal velocity (V_{max}) and Michaelis-Menten constant (K_m), were evaluated at pH 8.5 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.

3.3.11 Determination of protein content

Protein concentration of all samples was measured by the method of Lowry *et al*. (1951), except for ion-exchange fraction, which was measured by the method of Bradford (1976). Bovine serum albumin was used as a standard for both assays.

3.3.12 Statistical analysis

Experiments were run in triplicate. All data were subjected to analysis of variance (ANOVA) and differences between means were evaluated by Duncan's Multiple Range Tests (Steel and Torrie, 1980). SPSS Statistic Program (Version 10.0) (SPSS Inc, Chicago, IL, USA) was used for data analysis.

3.4 Results and Discussion

3.4.1 Purification of trypsin from pyloric caeca of brownstripe red snapper

Trypsin was purified from the pyloric caeca of brownstripe red snapper by a series of chromatographies after precipitation with ammonium sulfate (40-60% saturation) as summarised in Table 7. An 8-fold increase in purity was obtained by ammonium sulfate precipitation. Ammonium sulfate precipitation was a simple method and generally introduced as an initial step to remove other proteins from the crude extract. Hau and Benjakul (2006) found that ammonium sulfate (40-60% saturation) precipitation of trypsin from pyloric caeca of bigeye sapper resulted in a 4.53-fold increase in purity. ASF was further purified using SBTI-Sepharose 4B affinity column. This purification step resulted in an increase in purity of 13-fold, compared with that of PCE, and a yield of 16.0% was obtained. Two protein bands of SBTI-affinity fraction were observed (Figure 12A; lane A). SBTI-Sepharose 4B affinity chromatography was performed in the first step of purification of trypsin from Atlantic bonito (*Sarda sarda*) pyloric caeca with the yield 20.7% and 22.4-fold increase in purity and gel-filtration chromatography was further used to obtain the purity of 47.6-fold with 11.9% yield. (Klomkloa *et al*., 2007a). However, trypsins were purified from the pyloric caeca of chinook salmon (Kurtovic *et al*., 2006), bigeye snapper (Hau and Benjakul, 2006) and New Zealand hoki (*Macruronus Novaezealandlae*) (Shi *et al*., 2007), using only SBTI-Sepharose 4B affinity chromatography, in which increases in purity by 6.5-, 34.0- and 44.6-fold, respectively and the yields of 2.7%, 13% and 23.9% were obtained, respectively.

Table 7. Purification of trypsin from pyloric caeca of brownstripe red snapper.

Purification steps	Total protein (mg)	Total activity (unit*)	Specific activity (unit/mg)	Yield (%)	Purity (fold)
Crude extract	271.33	5,953.95	21.94	100	1
Ammonium sulfate fraction (40-60% saturated)	11.63	1,918.42	164.90	32	8
SBTI-affinity chromatography	3.37	954.45	283.10	16	13
DEAE chromatography	0.67	289.15	434.72	5	20

*One unit was defined as that releasing 1 nmol of *p*-nitroaniline per min

SBTI-affinity fraction was subsequently subjected to a DEAE-Sephacel column. After elution, using a linear gradient of 0-0.5 M NaCl, the active fractions were pooled (Figure 11B). Only one protein band was found in the DEAE-Sephacel fraction when determined using SDS-PAGE (Figure 12A; lane 1). A purity of 20-fold was obtained for the DEAE-Sephacel fraction. Ion-exchange chromatography, especially DEAE ion-exchange chromatography, was used to remove the contaminating proteins and to purify different trypsins from the pyloric caeca of starfish (*Asterina Pectinifera*) (Kishimura and Hayashi, 2002) and from the spleen of yellowfin tuna (*Thunnus albacores*) (Klomklao *et al*., 2006b) and skipjack tuna (*Katsuwonus pelamis*) (Klomklao *et al*., 2007b). Trypsin from pyloric caeca of chum salmon (*Oncorhynchus keta*) was purified to homogeneity with 20.4-fold purity using affinity chromatography, gel-filtration, and DEAE ion-exchange chromatography (Sekizaki *et al*., 2000). Ahsan and Watabe (2001) purified two trypsins from viscera of Japanese anchovy (*Engraulis japonicus*) using affinity chromatography, gel-filtration, and DEAE ion-exchange chromatography. Purities of 30- and 58-fold were achieved for trypsin I and trypsin II, respectively.

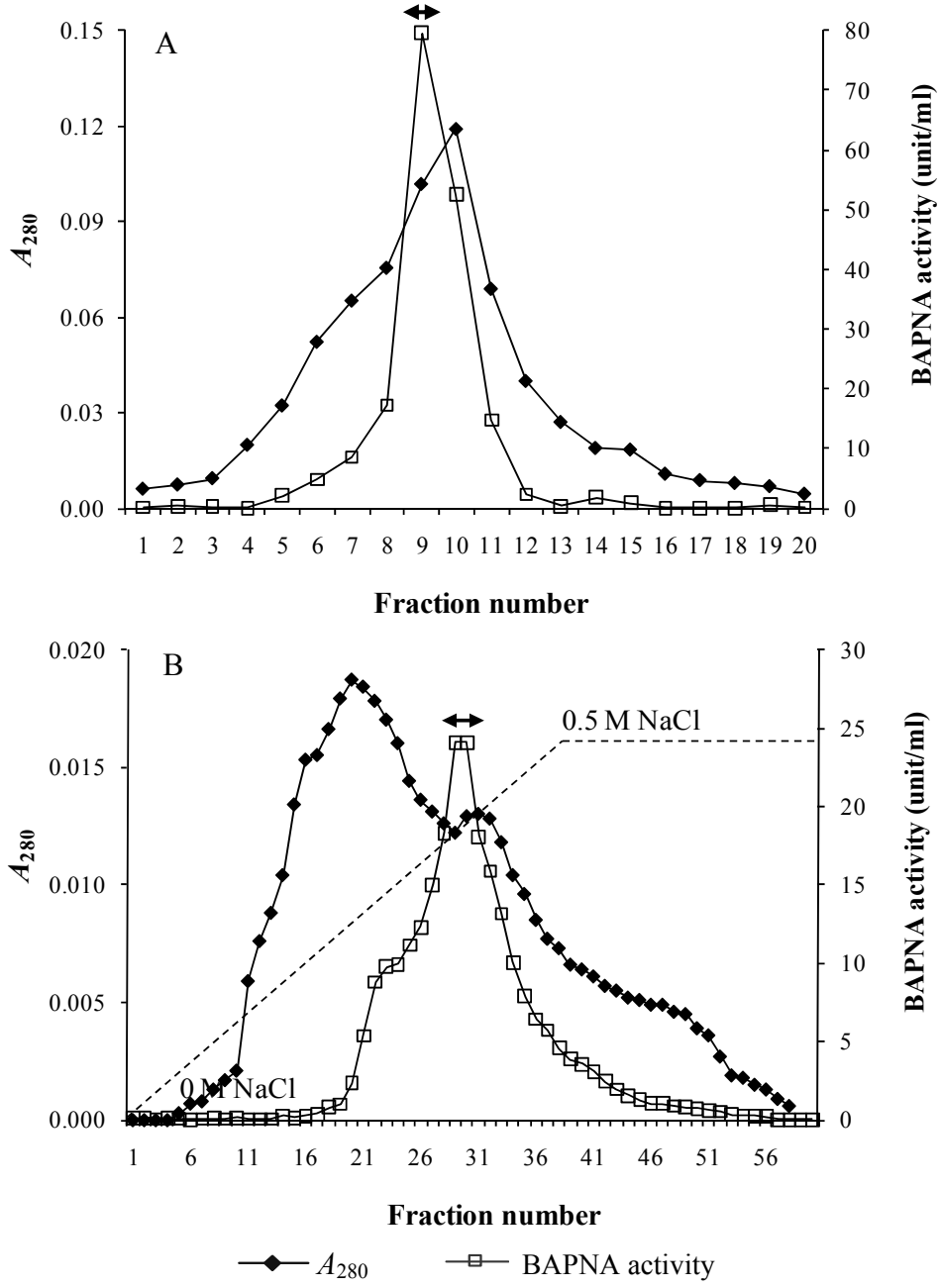

Figure 11. Elution profile of ammonium sulfate (40-60% saturation) fraction on SBTI-Sepharose 4B column (A) and elution profile of SBTI-Sepharose 4B fraction on DEAE-Sephacel column (B). Elution was performed using 5 mM HCL with a flow rate of 1.0 mL/min for SBTI-Sepharose 4B column and using a gradient (0-0.5 M NaCl) with a flow rate of 0.5 mL/min for DEAE-Sephacel column. Fractions (2 mL) were determined for trypsin activity using BAPNA as a substrate. (↔): pooled fractions.

The DEAE-Sephacel fraction showed a single band when determined using SDS-PAGE. The apparent molecular weight (MW) of the purified trypsin was estimated to be approximately 23 kDa (Figure. 12A). Based on native-PAGE (Figure 2B), this fraction also showed a single protein band, reconfirming that the purified trypsin had only one isoform. Different MWs of trypsins from various fish were reported, such as 21 and 21.5 kDa for mandarin fish (Lu *et al*., 2008), 24 kDa for arabesque greenling and walleye pollock (Kishimura *et al*., 2006; Kishimura *et al*., 2008), 25 kDa for Monterey sardine (Castillo-Yáñez *et al*., 2005), 26 kDa for New Zealand hoki (Shi *et al*., 2007), 28 kDa for bluefish (*Pomatomus saltatrix*) and chinook salmon (Klomkloa *et al*., 2007d; Kurtovic *et al*., 2006) and 38.5 kDa for tambaqui (Bezerra *et al*., 2001). The different MWs among trypsins from different sources may be due to genetic variation among species. Generally, trypsins have been reported to have molecular weights between 20 and 30 kDa (Lu *et al*., 2008).

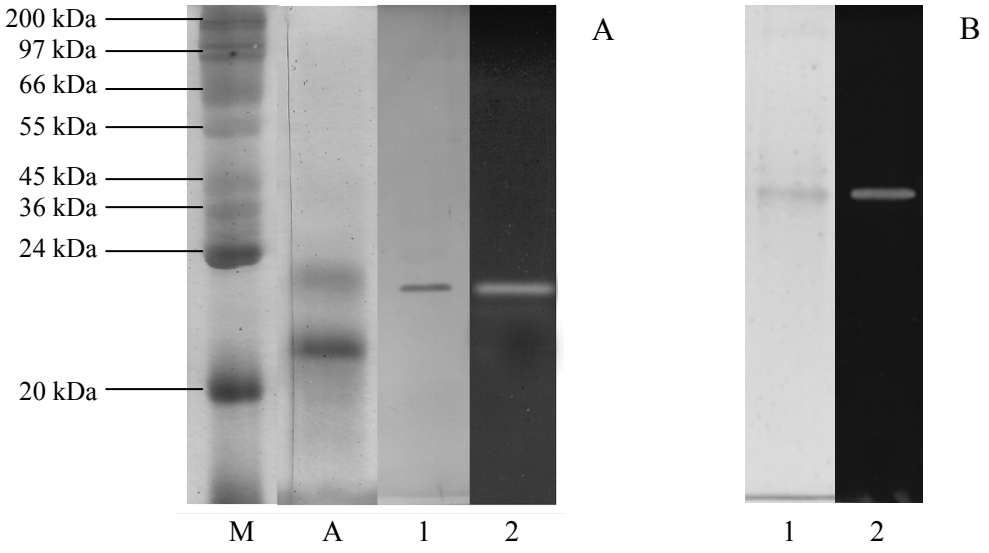

Figure 12. SDS-PAGE (A) and native-PAGE (B) of purified trypsin from pyloric caeca of brownstripe red snapper. Lane A: Sepharose 4B fraction. Lanes 1 and 2: protein band and activity band of DEAE-Sephacel fraction, respectively. M: molecular weight marker.

3.4.2 Effects of various protease inhibitors on the activity of trypsin from pyloric caeca of brownstripe red snapper

Protease inhibitors are very important tools for classify the types of proteases (Kishimura *et al*., 2006). Table 8 shows the effects of various protease inhibitors on the activity of the purified trypsin. Trypsin from pyloric caeca of brownstripe red snapper was almost inhibited (99.2-99.6% inhibition) by soybean trypsin inhibitor (a serine proteinase inhibitor) and TLCK (a trypsin specific inhibitor). While E-64, *N*-ethylmaleimide and iodoacetic acid (cysteine proteinase inhibitors), pepstatin A (an aspartic proteinase), EDTA (a metalloproteinase) and TPCK (a chymotrypsin specific inhibitor) had very small inhibitory effects on trypsin activities (0.2-1.4%). The result indicated that the protease purified from pyloric caeca of brownstripe red snapper was most likely a trypsin.

Table 8. Effects of various protease inhibitors on inhibition of trypsin from brownstripe red snapper*

Inhibitors	Final concentration	% Inhibition
Control	-	-
E-64	0.1 mM	0.5
N-ethylmaleimide	1 mM	1.0
Iodoacetic acic	1 mM	0.3
SBTI	1 g/L	99.6
TLCK	5 mM	99.2
TPCK	5 mM	0.9
EDTA	2 mM	1.4
Pepstatin A	0.01 mM	0.2

*The residual activities of trypsin after a 15 min incubation in the presence of inhibitors were assayed at 60^{o}C and pH 8.5 using BAPNA as substrate.

3.4.3 Effects of temperature and pH on the activity of trypsin from pyloric caeca of brownstripe red snapper

Temperature and pH profiles of purified trypsin from pyloric caeca of brownstripe red snapper are shown in Figure 13(a) and (b), respectively. The optimal temperature for BAPNA hydrolysis was 60°C. However, the activity markly decreased at temperatures above 60°C, mainly due to thermal denaturation of trypsin (Khantaphant and Bejakul, 2008) (Chapter 2). The same optimal temperature (60°C) of trypsin from menhaden (*Brevoortiu* spp) and mullet (*Mugil* spp) (Pavlisko *et al*., 1999), Japanese anchovy (Ahsan and Watabe, 2001), chinook salmon (Kurtovic *et al*., 2006) and New Zealand hoki (Shi *et al*., 2007) were reported when BAPNA was used as a substrate. Trypsin from brownstripe red snapper had an optimal temperature higher than those reported for cold-water fish trypsins, which had optimal temperatures in the range 40-45°C (Simpson, 2000). The differences might be associated with different living temperatures of fish (Kishimura *et al*., 2008) as well as differences in substrates or assay conditions used (Klomklao *et al*., 2007a).

The maximal activity of trypsin from brownstripe red snapper was observed at pH 8.5. Marked loss in activity was found at pH 4.0. Furthermore, a decrease in activity was noticeable at very alkaline pHs. Trypsins from pyloric caeca of bigeye snapper and chinook salmon had optimal pHs of 8.0 (Hau and Benjakul, 2006; Kurtovic *et al*., 2006). Trypsin from spotted goatfish had an optimal pH of 9.0 (Souza *et al*., 2007); Japanese anchovy and tambaqui trypsins showed the maximal activity at pH 9.5 (Ahsan and Watabe, 2001; Bezerra *et al*., 2001). Simpson (2000) reported that trypsins are generally more active at alkaline pH with pH optimal ranging from 7.5 to 10.5.

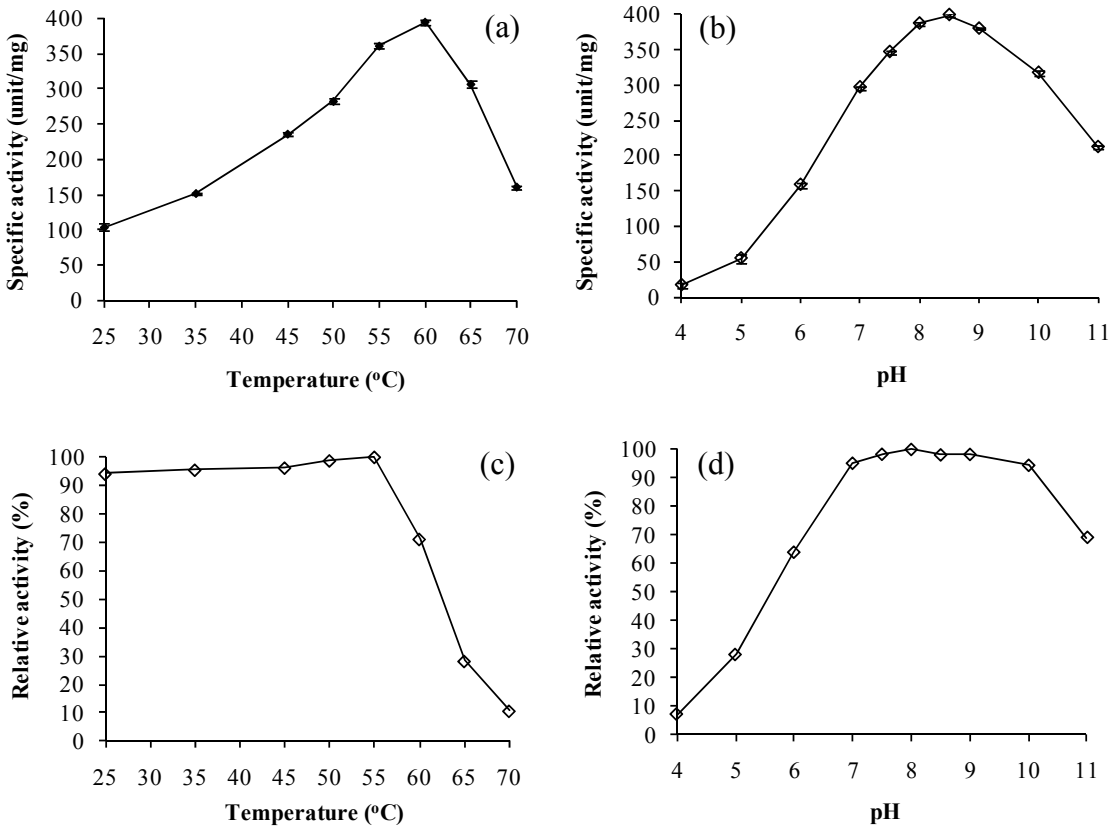

Figure 13. Temperature (a) and pH (b) profiles and thermal (c) and pH (d) stability of purified trypsin from pyloric caeca of brownstripe red snapper. For temperature profile, the activity of trypsin was determined at pH 8.0 using BAPNA as substrate. pH profile was studied at 60°C at various pHs. For thermal and pH stability, the remaining activity was assayed at 60°C and pH 8.5 using BAPNA as substrate.

3.4.4 Thermal and pH stability of purified trypsin from pyloric caeca of brownstripe red snapper

Thermal and pH stability of purified trypsin from brownstripe red snapper is depicted in Figure 13(c) and (d), respectively. Trypsin was quite stable up to 55°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. Trypsin from brownstripe red snapper was more resistant to high temperature than were trypsins from bluefish, chinook salmon and New Zealand hoki, which lost their activity rapidly at temperatures above 40 °C (Klomklao *et al*., 2007d; Kurtovic *et al*., 2006; Shi *et al*., 2007). Furthermore, Bezerra *et al*. (2005) found that trypsin from Nile tilapia (*Oreochromis niloticus*) was stable up to 50°C and similar thermal stability was reported for trypsins from Japanese anchovy (Ahsan and Watabe, 2001) and mullet (Pavlisko *et al*., 1999). In general, thermal stability of fish trypsin varies with species as well as with incubation conditions (Shahidi and Kamil, 2001).

For pH stability, trypsin from brownstripe red snapper showed high stability over neutral and alkaline pH ranges (pH 7.0-10.0). A sharp decrease in activity was more pronounced in the acidic pH range. This indicated the irreversible denaturation of trypsin at acidic pHs (Klomklao *et al*., 2007d). However, a slight decrease in activity was found at pH above 10.0. Trypsin from menhaden and mullet (Pavlisko *et al*., 1999), bigeye snapper (Hau and Benjakul, 2006), New Zealand hoki (Shi *et al*., 2007), bluefish (Klomklao *et al*., 2007d) and Atlantic bonito (Klomklao *et al*., 2007a) were stable in the neutral and alkaline pH ranges, in which 80-100% of activity was retained. Shahidi and Kamil (2001) concluded that fish trypsins were stable in the alkaline pH range.

3.4.5 Kinetic study

Kinetic data for purified trypsin from brownstripe red snapper are summarised in Table 9. K_m and k_{cat} for the hydrolysis of BAPNA were 0.51 mM and 4.71 s^{-1}, respectively, and the corresponding values for the hydrolysis of TAME were 0.33 mM and 112 s^{-1}, respectively. The catalytic efficiencies (k_{cat} / K_m) for the hydrolysis of BAPNA and TAME were calculated to be 9.3 and 341 $s^{-1}mM^{-1}$, respectively. The result indicated that trypsin from brownstripe red snapper had a higher affinity for TAME than did BAPNA.

Table 9. Kinetic parameters of brownstripe red snapper trypsin and other fish trypsins.

Substrates	Trypsins	K_m* (mM)	k_{cat}* (s^{-1})	k_{cat} / K_m ($s^{-1}mM^{-1}$)
BAPNA	Brownstripe red snapper (*Lutjanus vitta*)	0.51	4.71	9.3
	New Zealand hoki (*Macruronus Novaezealandlae*) [a]	0.06	0.33	5.5
	Chum salmon (*Oncorhynchus keta*) [b]	0.03	2.29	79.0
	Anchovy (*Engraulis japonica*) [c]	0.05	1.52	30.7
	Monterey sardine (*Sardinops sagax caerulea*) [d]	0.05	2.12	41.0
	Bigeye snapper (*Priacanthus macracanthus*) [e]	0.31	1.06	3.4
TAME	Brownstripe red snapper (*Lutjanus vitta*)	0.33	112	341
	New Zealand hoki (*Macruronus Novaezealandlae*) [a]	2.08	19	9.1
	Chum salmon (*Oncorhynchus keta*) [b]	0.003	72.35	24,117
	Anchovy (*Engraulis japonica*) [c]	0.84	39.67	47.2
	Tongol tuna (*Thunnus tonggol*) [f]	0.25	200	800

* K_m and k_{cat} values of all trypsins were determined at 30°C under optimal pH, except trypsins from New Zealand hoki and chum salmon, which were assayed at 25°C.
[a] Shi *et al*. (2007); [b] Sekizaki *et al*. (2000); [c] Heu *et al*. (1995); [d] Castillo-Yañez *et al*. (2005); [e] Hau and Benjakul (2006); [f] Klomklao *et al*. (2007c)

The kinetic parameters of trypsin from brownstripe red snapper were also compared with those of other fish species (Table 9). For the BAPNA hydrolysis reaction, trypsin from brownstripe red snapper had a higher K_m values, than had trypsins from New Zealand hoki (Shi *et al*., 2007), chum salmon (Sekizaki *et al*., 2000), anchovy (*Engraulis japonica*) (Heu *et al*., 1995), Monterey sardine (Castillo-Yañez *et al*., 2005) and bigeye snapper (Hau and Benjakul, 2006). K_m is often associated with the affinity of the enzyme for substrate (Engel, 1977). Therefore, trypsin from brownstripe red snapper had a lower affinity for BAPNA than had those from other fish species. For TAME hydrolysis, a lower K_m was observed for trypsin from brownstripe red snapper, than those from New Zealand hoki (Shi *et al*., 2007) and anchovy (Heu *et al*., 1995). This indicated that trypsin from brownstripe red

snapper had a higher affinity for TAME than had trypsins from New Zealand hoki and anchovy.

Purified trypsin from pyloric caeca of brownstripe red snapper showed the higher k_{cat} than did those from other fish species when BAPNA was used as a substrate. For the TAME hydrolysis reaction, trypsin from brownstripe red snapper also showed a higher k_{cat} than did those from New Zealand hoki (Shi *et al*., 2007), chum salmon (Sekizaki *et al*., 2000) and anchovy (Heu *et al*., 1995). However, k_{cat} of trypsin from tongol tuna (*Thunnus tonggol*) spleen (Klomklao *et al*., 2007c) was higher than that of trypsin from brownstripe red snapper. k_{cat} indicates the maximum number of enzymatic reactions catalysed per second (Fersht, 1977). Thus, the higher k_{cat} of trypsin suggests a higher rate of substrate hydrolysis.

Catalytic efficiency (k_{cat} / K_m) of trypsin from brownstripe red snapper was lower than those from chum salmon (Sekizaki *et al*., 2000), anchovy (Heu *et al*., 1995) and Monterey sardine (Castillo-Yañez *et al*., 2005) when BAPNA was used as a substrate. A lower k_{cat} / K_m was also observed with trypsin from chum salmon (Sekizaki *et al*., 2000) and from tongol tuna (Klomklao *et al*., 2007c) when TAME was used as a substrate. However, trypsin from brownstripe red snapper showed a higher catalytic efficiency than did trypsins from New Zealand hoki and bigeye snapper when BAPNA was used as a substrate. A higher catalytic efficiency was noticeable, compared with those of New Zealand hoki and anchovy when TAME was used as a substrate. The higher catalytic efficiency, the more efficient is the enzyme in transforming the substrate to product (Fersht, 1977). The result suggests that trypsin from brownstripe red snapper was more effective in hydrolysis of BAPNA than were trypsins from New Zealand hoki and bigeye snapper and it also exhibited higher hydrolytic activity toward TAME in comparison with trypsin from New Zealand hoki and anchovy.

3.5 Conclusion

Based on the ability to hydrolyse specific synthetic substrates, molecular weight, optimal condition of reaction and the response to specific trypsin inhibitor, the purified protease from pyloric caeca of brownstripe red snapper was classified as trypsin. Trypsin exhibited the highest hydrolytic activity toward BAPNA at 60°C and pH 8.5. Therefore, pyloric caeca may be an important source of trypsin, for further used.

CHAPTER 4

THE EFFECTS OF PRETREATMENTS ON ANTIOXIDATIVE ACTIVITIES OF PROTEIN HYDROLYSATE FROM THE MUSCLE OF BROWNSTRIPE RED SNAPPER

4.1 Abstract

Different pretreatments of mince from brownstripe red snapper (*Lutjanus vitta)* including 1) washing; 2) membrane separation; 3) washing followed by membrane separation and 4) membrane separation followed by washing were conducted prior to hydrolysis. Among the resulting minces, that subjected to membrane separation with subsequent washing (MS/W) contained the lowest remaining myoglobin content, phospholipid content, heme iron and non-heme iron contents ($p < 0.05$) and showed the lowest TBARS values throughout 9 days of storage at 4°C in the presence and absence of 0.15 M cupric acetate ($p < 0.05$). When hydrolysates from 1) mince, 2) MS/W and 3) protein isolate from MS/W (PI) with different degree of hydrolysis (DH) (20, 30 and 40%) were prepared using proteases from pyloric caeca of brownstripe red snapper, antioxidative activities determined by DPPH, ABTS radical scavenging activities, ferric reducing antioxidant power and metal chelating activity varied with hydrolysates and DH. Antioxidative activities increased with increasing DH up to 40% ($p < 0.05$). At all DH tested, hydrolysate prepared from MS/W exhibited the highest antioxidative activities determined by all assays, compared to those from mince and PI ($p < 0.05$). Hydrolysate from MS/W with 40% DH had the molecular weight lower than 6.5 kDa as determined by SDS-PAGE. In liposome oxidation system, the addition of hydrolysate from MS/W resulted in the lower TBARS, compared with the control throughout the incubation period of 48 h at room temperature (25-28°C). Therefore, fish mince with membrane separation followed by washing was the most appropriate source for production of hydrolysate possessing antioxidative activity with the lowered amount of lipids and pro-oxidants.

4.2 Introduction

Numerous fish protein hydrolysates have been shown to have antioxidative activities such as gelatin hydrolysate from the skin of Alaska pollack (Kim *et al*., 2001), protein hydrolysate from the frame of yellowfin sole (Jun *et al*., 2004), protein hydrolysate from the muscle of round scad (Thiansilakul *et al*., 2007a) and yellow stripe trevally (Klompong *et al*., 2007). One critical problem of hydrolysate preparation from these proteinaceous sources is the presence of pro-oxidants such as heme proteins and unstable lipid substrates (Raghavan and Kristinsson, 2008). As a consequence, the desirable antioxidative activity can be lowered and the hydrolysate may exhibit the pro-oxidative activity to some extent.

Fish muscle contains myoglobin and other heme proteins, which in turn become the major pro-oxidants in muscle (Chaijan *et al*., 2005; Thanonkaew *et al*., 2006). Furthermore, phospholipids, major components of cell membranes, are believed to readily prone to oxidative deterioration due to its highly unsaturated fatty acid composition (Borst *et al*., 2000). In fish, especially lean fish, phospholipids make up most of the lipids of the cell (Liang and Hultin, 2005a). The presence of these pro-oxidants and lipids could decrease the stability of protein hydrolysates and may limit their use in food systems (Raghavan and Kristinsson, 2008). Removal of these compounds could therefore alleviate such problems occurring in hydrolysate from fish muscle.

Brownstripe red snapper (*Lutjanus vitta*), a lean fish, is one of the main raw materials for surimi production in Thailand (Khantaphant and Benjakul, 2008) (Chapter 2). Apart from being processed into surimi, its flesh can be used as the raw material for production of protein hydrolysate with bioactivities. To produce protein hydrolysate from brownstripe red snapper with high antioxidative activity and negligible pro-oxidant or lipids susceptible to oxidation, the appropriate pretreatment of muscle by washing and/or membrane separation before hydrolysis should therefore taken into consideration.

Pyloric caeca of various fish has been reported to be the important source of trypsin such as arabesque greenling (Kishimura *et al*., 2006), New Zealand

hoki (Shi *et al*., 2007) and bluefish (Klomklao *et al*., 2007d). Recently, protease from pyloric caeca of brownstripe red snapper has been shown to hydrolyze fish skin gelatin effectively (Khantaphant and Benjakul, 2008) (Chapter 2). Along with the pretreatment, proteases from pyloric caeca of brownstripe red snapper could be used as the potential aid for the preparation of protein hydrolysate with antioxidative activity, in which the new value-added product of health benefit could be produced. The objectives of this study were to elucidate the effect of washing and membrane separation on the removal of pro-oxidant and membranes and to determine the antioxidative activity of protein hydrolysates from pretreated mince of brownstripe red snapper prepared using proteases from its pyloric caeca.

4.3 Materials and Methods

4.3.1 Chemicals

2,2′-azinobis(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS), 1,1,3,3-tetramethoxypropane, bathophenanthroline disulfonic acid, 1,1-diphenyl-2-picrylhydrazyl (DPPH), 2,4,6-tripyridyl-triazine (TPTZ) and 3-(2-pyridyl)-5,6-diphenyl-1,2,4-triazine-4′,4″-disulfonic acid (ferrozine) were purchased from Sigma (St. Louis, MO, USA). Thiobarbituric acid (TBA) and potassium persulfate were obtained from Fluka (Buchs, Switzerland). Iron standard solution and sodium nitrite were procured from Merck (Darmstadt, Germany). Sodium sulfite and ammoniumthiocyanate were obtained from Riedel-deHaën (Seelze, Germany). All chemicals were of analytical grade.

4.3.2 Preparation of mince with different pretreatments

4.3.2.1 Mince preparation

Brownstripe red snapper, stored in ice and off-loaded approximately 24-36 days after capture, were purchased from a dock in Songkhla province, Thailand. Fish were transported in ice with the fish/ice ratio of 1:2 (w/w) to the

Department of Food Technology, Prince of Songkla University within 1 h. Upon arrival, whole fish were washed and only flesh was separated manually. Flesh was minced to uniformity using Moulinex AY46 blender (Group SEB, Lyon, France). The mince obtained was placed in polyethylene bags and kept in ice not longer than 2 h before use.

4.3.2.2 Preparation of washed mince

Mince was homogenized with five volumes of cold distilled water using an IKA Labortechnik homogenizer (Selangor, Malaysia) at a speed of 11000 rpm for 2 min, followed by stirring at 4°C for 15 min prior to centrifuging at 9600 x *g* for 10 min at 4°C using a Beckman Coulter centrifuge Model Avant J-E (Beckman Coulter, Inc., Fullerton, CA, USA). The washing process was repeated twice. The sample obtained was referred to as 'washed mince; W'.

4.3.2.3 Preparation of mince with membrane separation

Membrane was removed from mince by treatment with Ca^{2+} and citric acid according to the method of Liang and Hultin (2005b) with a slight modification. Mince was homogenized with nine volumes of cold 8 mM $CaCl_2$ solution in the presence of 5 mM citric acid using a homogenizer at a speed of 11,000 rpm for 2 min. After continuous stirring for 60 min at 4°C, the sample was centrifuged at 4,000 x *g* for 15 min at 4°C and the mince obtained was referred to as 'membrane separated mince; MS'.

4.3.2.4 Preparation of membrane separated/washed mince and washed/membrane separated mince

Minces subjected to washing prior to membrane separation (W/MS) and to membrane separation followed by washing (MS/W) were also prepared. All samples were subjected to analyses.

4.3.3 Analyses

4.3.3.1 Determination of myoglobin content

Myoglobin content was determined by direct spectrophotometric measurement (Chaijan *et al*., 2005). Sample (2.0 g) was mixed with 20 mL of cold 40 mM phosphate buffer (pH 6.8), followed by homogenization at 13,500 rpm for 10 s. The mixture was centrifuged at 3,000 x *g* for 30 min at 4°C and the supernatant was filtered through Whatman filter paper No. 1 (Whatman International Ltd., Maidstone, England). The absorbance of supernatant was read at 525 nm. Myoglobin content was calculated from the molar extinction coefficient of 7.6 x 10^{-3} and a molecular weight of 16,110 (Gomez-Basauri and Regenstein, 1992). The myoglobin content was expressed as mg/100g dry sample.

4.3.3.2 Determination of heme iron and non-heme iron contents

Heme iron content was calculated based on myoglobin content, which contains iron at a level of 0.35 % (Gomez-Basauri and Regenstein, 1992). Heme iron content was expressed as mg/100g dry sample.

Non-heme iron content was determined according to the method of Chaijan *et al*. (2005). Sample (1.0 g) was weighed and transferred into a screw cap test tube and 50 mL of 0.39 % (w/v) sodium nitrite and 4 ml of a mixture of 40 % TCA and 6 M HCl (1:1 (v/v) ratio, freshly prepared) were added. The tightly capped tubes were placed in a temperature controlled water bath (Memmert Model W350, Schwabach, Germany) for 22 h at 65°C followed by cooling to room temperature (25-28°C) for 2 h. The supernatant (400 µL) was mixed with 2 mL of the non-heme iron color reagent, a freshly prepared mixture of bathophenanthroline disulfonic acid, double-deionized water and saturated sodium acetate solution at a ratio of 1:20:20 (w/v/v). After thorough mixing, the mixture was allowed to stand for 10 min and the absorbance was measured at 540 nm. The non-heme iron content was calculated from the iron standard curve using iron standard solution (0-2 ppm). The non-heme iron content was expressed as mg/100g dry sample.

4.3.3.3 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 extracted from the sample by the method of Bligh and Dyer (1959). Thereafter, lipids (20 μL) were dissolved in chloroform to 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 with phosphatidylcholine (0-0.1 ppm). The phospholipid content was expressed as mg/100g dry sample.

4.3.4 Occurrence of lipid oxidation in mince with different pretreatments

Mince with different pretreatments was mixed with sodium azide to obtain a final concentration of 0.01 % (w/w). The mixture (50 g) was added with 20 μL of 0.15 M cupric acetate and the samples were stored at 4°C and taken every 3 days for determination of lipid oxidation.

4.3.4.1 Determination of thiobarbituric acid reactive substances

Thiobarbituric acid reactive substances (TBARS) were determined as described by Benjakul *et al*. (2005a) with a slight modification. Sample was homogenized with TBARS solution (0.375 % TBA, 15 % TCA and 0.25 M HCl) with a ratio of 1:4 (w/v). 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 5,000 x *g* for 10 min at room temperature using Hettich centrifuge (Model MIKRO-20, Hettich, Tuttlingen, Germany). The supernatant was collected and measured at 532 nm using a UV-1601 Spectrophotometer (Shimadzu, Kyoto, Japan). TBARS was calculated from a standard curve of malonaldehyde (MDA) (0-10 ppm) and expressed as mg MDA/kg sample.

4.3.4.2 Determination of conjugated diene

Conjugated diene formed in liposome oxidation system was measured according to the method of Frankel *et al*. (1997). Sample (0.1 mL) was dissolved in methanol (5.0 mL) and conjugated dienes were measured as the increase in absorbance at 234 nm.

4.3.5 Dissociation of actomyosin complex in mince with different pretreatments

Mince with different pretreatments were determined for ATPase activity. Prior to assay, natural actomyosin (NAM) was prepared as described by Benjakul *et al*. (1997) with a slight modification. Pretreated mince was homogenized in cold 0.6 M KCl (pH 7.0) at a ratio of 1:10 (w/v) using a homogenizer with a speed of 11,000 rpm for 20 s, followed by a 20 s rest interval to a total extraction time of 4 min. Thereafter, the homogenate was centrifuged at 5,000 x *g* for 30 min at 4°C. Three volumes of cold distilled water were added to precipitate NAM prior to centrifugation at 5,000 x *g* for 20 min at 4°C. NAM pellet was dissolved in a minimal volume of cold 50 mM potassium phosphate buffer (pH 7.0) containing 0.6 M KCl. NAM solution was kept in ice and used within 1 h.

Mg^{2+}-ATPase activities of NAM were assayed according to the method of Benjakul *et al*. (2001). NAM solution (0.5 mL) was added into 0.3 mL of 0.5 M Tris-maleate buffer (pH 7.0) and 2 mM $MgCl_2$ was added to a mixture to a final volume of 9.5 mL. Thereafter, 0.5 mL of 20 mM ATP was added to initiate the reaction. The reaction was conducted at 25°C for exactly 10 min and 5 mL of cold 15 % TCA were added to terminate the reaction. The reaction mixture was centrifuged at 3,500 x *g* for 5 min and the inorganic phosphate liberated in the supernatant was measured as described by Fiske and Subbarow (1925). Specific activity was expressed as μmol inorganic phosphate released / mg protein / min. Blank was performed by adding the cold TCA prior to the addition of ATP. The decrease in Mg^{2+}-ATPase indicated the dissociation of actomyosin complex, in which actin was not stable in the presence of high salt concentration (Torigai and Konno, 1996).

4.3.6 Preparation of proteases from pyloric caeca

Pyloric caeca from brownstripe red snapper was collected and powered in liquid nitrogen. Thereafter, the pyloric caeca extract was prepared according to the method of Khantaphant and Benjakul (2010) (Chapter 3) with a slight modification. Pyloric caeca powder was suspended in ten volumes of extraction buffer (50 mM Tris-HCl buffer, pH 8.0 containing 10 mM $CaCl_2$). The mixture was homogenized using a homogenizer at 11,000 rpm for 2 min. The homogenate was continuously stirred for 30 min at 4°C and centrifuged at 8,000 x *g* for 30 min at 4°C. The supernatant was filtered through a Whatman filter paper No. 1. The filtrate obtained was further subjected to 40-60% saturation ammonium sulfate precipitation. After stirring at 4°C for 30 min, the mixture was centrifuged at 8,000 x *g* for 30 min at 4°C and the pellet obtained was dissolved in 50 mM Tris-HCl buffer, pH 8.0 followed by dialysis 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 'pyloric caeca extract; PCE'. PCE was determined for proteolytic activity using casein as a substrate under optimal condition (60°C and pH 8.5)

4.3.7 Preparation of brownstripe red snapper protein isolate and hydrolysates

Mince, mince subjected to membrane separation and washing (MS/W) and protein isolate (PI) were used for preparation of protein hydrolysate. PI was prepared as described by Rawdkuen *et al*. (2009) with a slight modification. MS/W mince was homogenized with nine volumes of cold distilled water at a speed of 11,000 rpm for 1 min. The homogenate was adjusted to pH 11.2 using 2 M NaOH. Homogenates was then centrifuged at 5,000 x *g* for 10 min at 4°C. The alkaline-soluble fraction was collected and adjusted to pH 5.5 (the isoelectric point of muscle proteins) using 2 M HCl, followed by centrifuging at 5,000 x *g* for 10 min at 4°C. The pellet was mixed with distilled water at a ratio of 1:2 (w/v) and the final pH was

adjusted to 7.0 with 2 M NaOH. The sample obtained was referred to as 'protein isolate; PI'.

To prepare protein hydrolysate, the samples were mixed with 50 mM Tris-HCl buffer, pH 8.5 to obtain the protein concentration of 2%. The mixtures were homogenized at a speed of 11,000 rpm for 1 min and the homogenates were pre-incubated at 60°C for 10 min. Hydrolytic reaction was started by adding PCE with different levels to gain the desirable degree of hydrolysis of 20, 30 and 40% following the method of Benjakul and Morrissey (1997). After 2 h of hydrolysis, the reactions were terminated by heating the mixture in boiling water for 10 min. The mixture was centrifuged at 2,000 x *g* at 4°C for 10 min and the supernatant was collected and referred to as 'protein hydrolysate'. All protein hydrolysates were determined for antioxidative activities.

4.3.8 Determination of antioxidative activities

4.3.8.1 DPPH radical scavenging activity

DPPH radical scavenging activity was determined according to the method of Khantaphant and Benjakul (2008) (Chapter 2). Sample solution (1.5 mL) was added with 1.5 mL of 0.1 mM DPPH in 95% ethanol. The mixture was allowed to stand for 30 min in dark at room temperature. The resulting solution was measured at 517 nm. The control was prepared in the same manner except that distilled water was used instead of the sample. The DPPH radical scavenging activity was calculated from Trolox standard curve and expressed as μmol Trolox equivalents (TE)/g protein.

4.3.8.2 ABTS radical scavenging activity

ABTS radical scavenging activity was determined as described by Re *et al*. (1999). ABTS radical ($ABTS^{\bullet+}$) was produced by reacting ABTS stock solution with 2.6 mM potassium persulfate at the ratio of 1:1 (v/v). The mixture was allowed to react in dark for 12 h at room temperature. Prior to assay, $ABTS^{\bullet+}$ solution was diluted with methanol to obtain an absorbance of 1.1 (±0.02) at 734 nm. To initiate the reaction, 150 μL of sample was mixed with 2.85 mL of $ABTS^{\bullet+}$ solution. The

absorbance was then read at 734 nm after 2 h dark incubation at room temperature. ABTS radical scavenging activity was expressed as μmol TE/g protein. The control was prepared in the same manner except that distilled water was used instead of the sample.

4.3.8.3 Ferric reducing antioxidant power (FRAP)

The ability of samples to reduce ferric ion (Fe^{3+}) was evaluated by the method of Benzie and Strain (1996). FRAP reagent (a freshly prepared mixture of 10 mM TPTZ solution in 40 mM HCl, 20 mM $FeCl_3.6H_2O$ solution and 300 mM acetate buffer, pH 3.6 (1:1:10 v/v/v)) (2.85 mL) was incubated at 37°C for 30 min prior to mixing with 150 μL of sample. The reaction mixture was allowed to stand in dark for 30 min at room temperature. Absorbance at 593 nm was read and FRAP was expressed as μmol TE/g protein. Distilled water was used instead of the sample and prepared in the same manner to obtain the control.

4.3.8.4 Ferrous chelating activity

Chelating activity of protein hydrolysate towards ferrous ion (Fe^{2+}) was measured by the method of Benjakul *et al*. (2005a) with a slight modification. Sample (200 μL) was mixed with 800 μL of distilled water. Thereafter, 0.1 mL of 2 mM $FeCl_2$ and 0.2 ml of 5 mM ferrozine were added. The mixture was allowed to react for 20 min at room temperature. The control was prepared in the same manner except that distilled water was used instead of the sample. The absorbance was then read at 562 nm. The standard curve of EDTA was prepared and ferrous chelating activity was expressed as μmol EDTA equivalents/g protein.

4.3.9 Determination of antioxidative activity in a lecithin liposome system

The antioxidative activity of protein hydrolysates in a lecithin liposome system was determined according to the method of Frankel *et al*. (1997) slightly modified by Thiansilakul *et al*. (2007a). Lecithin liposome system was prepared by suspending lecithin in deionized water at a concentration of 8 mg/mL.

The mixture was stirred with a glass rod followed by sonicating for 30 min in a sonicating bath (Elma Model S30H, Singen, Germany). Protein hydrolysate (3 mL) was added to the lecithin liposome system (15 mL) to a final concentration of 1,000 ppm followed by sonicating the liposome suspension for 2 min. To initiate the reaction, 20 mL of 0.15 M cupric acetate were added. The mixture was shaken at 120 rpm using a shaker (Heidolph Model Unimax 1010, Schwabach, Germany) at 37°C in dark. The control and systems containing 25 or 100 ppm BHT were also prepared. Oxidation in lecithin liposome systems was monitored at 6 h intervals by determining the formation of TBARS and conjugated dienes.

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

Samples were determined for protein pattern using SDS-PAGE (Laemmli, 1970) with 4% stacking gel and 10 and 20% separating gel for mince without and with hydrolysis, respectively. Samples 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 1:1 (v/v). Proteins (15 μg) were loaded onto the gel. The electrophoresis was run at a constant current of 15 mA per gel by a Mini-Protean II Cell apparatus (Bio-Rad, Hercules, CA, USA). The gels were fixed and 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. Wide range molecular weight marker was used to estimate the molecular weight.

4.3.11 Determination of protein concentration

Protein concentration was measured by the method of Lowry *et al*. (1951) using bovine serum albumin as a standard.

4.3.12 Statistical analysis

Experiments were run in triplicate. All data were subjected to analysis of variance (ANOVA) and differences between means were evaluated by Duncan's Multiple Range Test (Steel and Torrie, 1980). SPSS Statistic Program (Version 10.0) (SPSS Inc, Chicago, IL, USA) was used for data analysis.

4.4 Results and Discussion

4.4.1 Effect of different pretreatment on composition of mince

Mince from brownstripe red snapper with different pretreatments including wash mince (W), membrane separated mince (MS), washed/membrane separated mince (W/MS) and membrane separated/washed mince (MS/W) had the different compositions (Figure 14). All treatments resulted in the decreases in myoglobin, phospholipid, heme iron and non-heme iron contents, compared with the control (C) ($p < 0.05$). However, washing showed no profound effect on non-heme iron content ($p > 0.05$) (Figure 15(d)). In general, mince subjected to both washing and membrane separation (W/MS and MS/W) showed the higher decreases in myoglobin, phospholipid and heme iron, compared with those pretreated with only one process (W and MS) ($p < 0.05$). The result suggested that pretreatment by washing together with membrane separation was able to remove heme pigment as well as phospholipids from mince.

Washing is the process used for the removal of sarcoplasmic protein and pro-oxidative aqueous components naturally present in fish muscle (Chen *et al*., 1997; Sannaveerappa *et al*., 2007). Myoglobin in ordinary muscle of sardine and mackerel mince was removed by 23 and 75 % by washing process (Chaijan *et al*., 2004). Thus, washing could lower the myoglobin and heme iron effectively. Baxter and Skonberg (2008) reported that washing not only removed sarcoplasmic proteins, but also concentrated the myofibrillar proteins. After washing, mince had the same muscle structure with the reduced content of aqueous pro-oxidants and endogenous

triacylglycerols (Larsson *et al*., 2007). In addition, washing could lower the phospholipid content in the resulting mince. Due to the polar head of phospholipid associated with cell membrane, it was more likely leached out during washing to some extent. After washing, mince had the decreases in myoglobin, phospholipid, heme iron and non-heme iron by 49.56, 42.55, 49.56 and 3.24 %, compared with those found in the mince, respectively. When the mince was subjected to membrane separation, the lower content of phospholipid was obtained ($p < 0.05$).

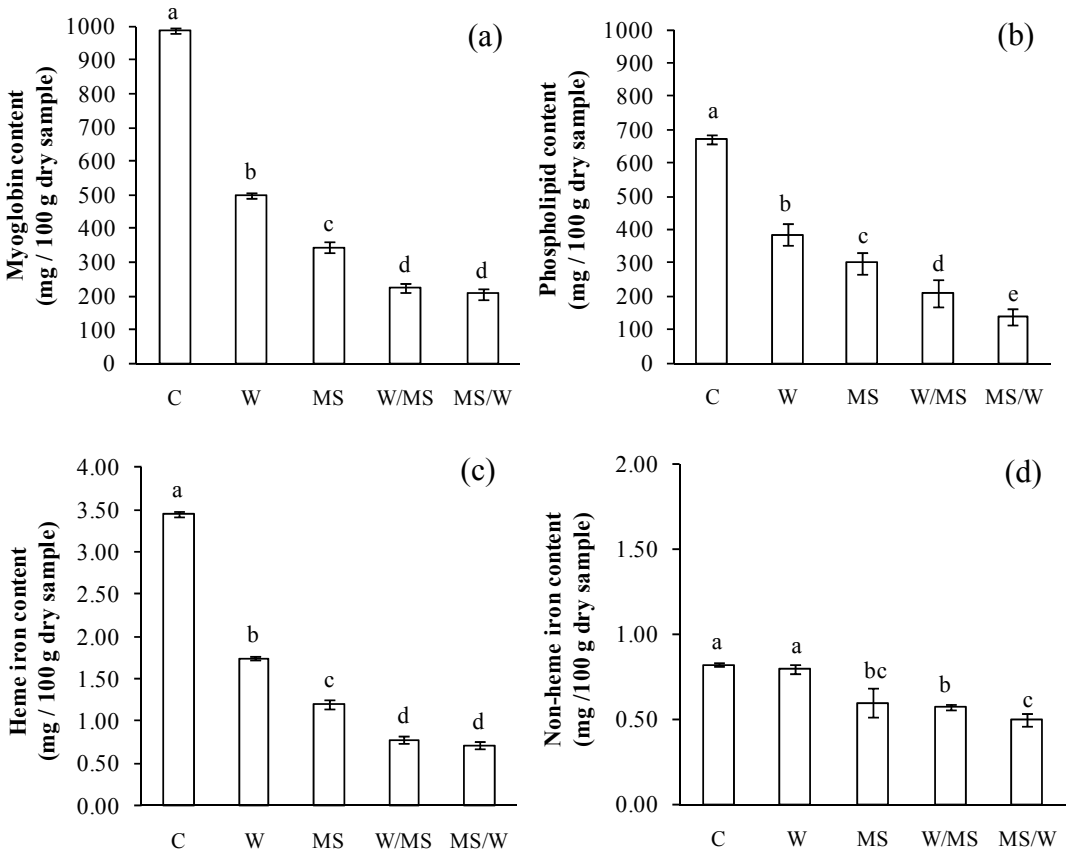

Figure 14. Myoglobin (a), phospholipid (b), heme-iron (c) and non-heme iron (d) contents of brownstripe red snapper mince with different pretreatments; W: washed mince, MS: membrane separated mince, W/MS: washed/membrane separated mince and MS/W: membrane separated/washed mince. Bars represent the standard deviation (n=3). Different letters indicate significant differences ($p < 0.05$).

In muscle tissue, phospholipid membranes are connected with cytoskeletal proteins through electrostatic attraction between the acidic phospholipids of membranes and the basic amino acid residues of the cytoskeletal proteins (Haleva *et al*., 2004). When mince was subjected to membrane separation using the solution containing Ca^{2+} ion and citric acid, the interaction between Ca^{2+} with the polar head of phospholipids as well as the competition between citric acid and the acidic phospholipids of membranes bound to the basic amino acid residues of cytoskeletal proteins more likely took place. As a result, the release of phospholipid membrane from attached cytoskeletal proteins occured. Liang and Hultin (2005a) reported that polycarboxylic groups of citric acid might compete with acidic phospholipids to bind with the basic amino acid residues of the cytoskeletal proteins, while Ca^{2+} induced the formation of calcium-phospholipid complex. Apart from the removal of phospholipids, the membrane separation process used also resulted in the lowering of myoglobin, heme iron and non-heme iron. Due to the polar nature of the medium used, myoglobin as well as iron could be soluble in the medium. Furthermore, Chen *et al*. (1997) reported that washing with cold water for 15 min eliminated 34% oil, compared with unwashed mince and 83% oil elimination was obtained when washing time of 135 min was used.

After membrane separation process, myoglobin, phospholipid, heme and non-heme iron were removed by 65.04, 55.33, 65.04 and 26.97 %, compared to those found in mince. When both washing and membrane separation processes were applied, all components tested decreased to a higher extent, compared to those found in mince with only one pretreatment or mince without pretreatment. Therefore, both processes exhibited the synergistic effect on removal of pro-oxidant and phospholipid membrane. It was noted that the sample with membrane separation, followed by washing had the lower phospholipid and non-heme iron than those with prior washing and membrane separation ($p < 0.05$). However, no differences in myoglobin and heme iron contents were noticeable between both samples ($p > 0.05$). Therefore, membrane separation/washing process was the appropriate pretreatment as evidenced by the lowest pro-oxidants and phospholipids in prepared mince.

4.4.2 Lipid oxidation in mince with different pretreatments

The remaining pro-oxidants and phospholipid membrane in mince might accelerate the oxidative reaction during handling or storage. During 9 days of storage at 4°C, TBARS of mince with different pretreatments in the presence and absence of Cu^{2+} are depicted in Figure 15. In the absence of Cu^{2+} (Figure 15(a)), TBARS in all pretreated samples increased as the storage time increased up to 6 days ($p < 0.05$). Nevertheless, TBARS of the control sample had the continuous increase in TBARS throughout the storage time of 9 days ($p < 0.05$). At day 9, TBARS of the control was almost 3-fold higher than that of pretreated mince. At the same storage time, the control showed the higher TBARS, compared with other samples with pretreatment ($p < 0.05$). The pronounced formation of TBARS in mince indicated the susceptibility of mince, which might be associated with the high content of phospholipid membrane with high PUFA as well as pro-oxidants. Among different pretreated mince, MS/W showed the lowest TBARS value, compared with other samples during 3-9 days of storage ($p < 0.05$). The lowest TBARS of MS/W was in agreement with the lowest remaining phospholipid and other pro-oxidative components (Figure 14).

In the presence of Cu^{2+} (Figure 15(b)), all samples showed the higher TBARS, in comparison with those without Cu^{2+}. Cu^{2+} has been used as the catalyst for lipid oxidation in different systems (Decker *et al*., 1992; Thanonkaew *et al*., 2006). The oxidation rates of all samples added with Cu^{2+} were enhanced as shown by higher TBARS. Thanonkaew *et al*. (2006) studied the effects of metal ions (Fe^{2+} and Cu^{2+}) addition to cuttlefish paste and found that the metal ions could accelerate lipid oxidation. Moreover, the remaining pro-oxidants in mince might accelerate the oxidative deterioration of lipids, particularly phospholipids with high PUFA content. The similar results were obtained, compared to those without Cu^{2+}, in which MS/W containing the lowest phospholipids had the lowest TBARS throughout the storage time, compared with other samples ($p < 0.05$).

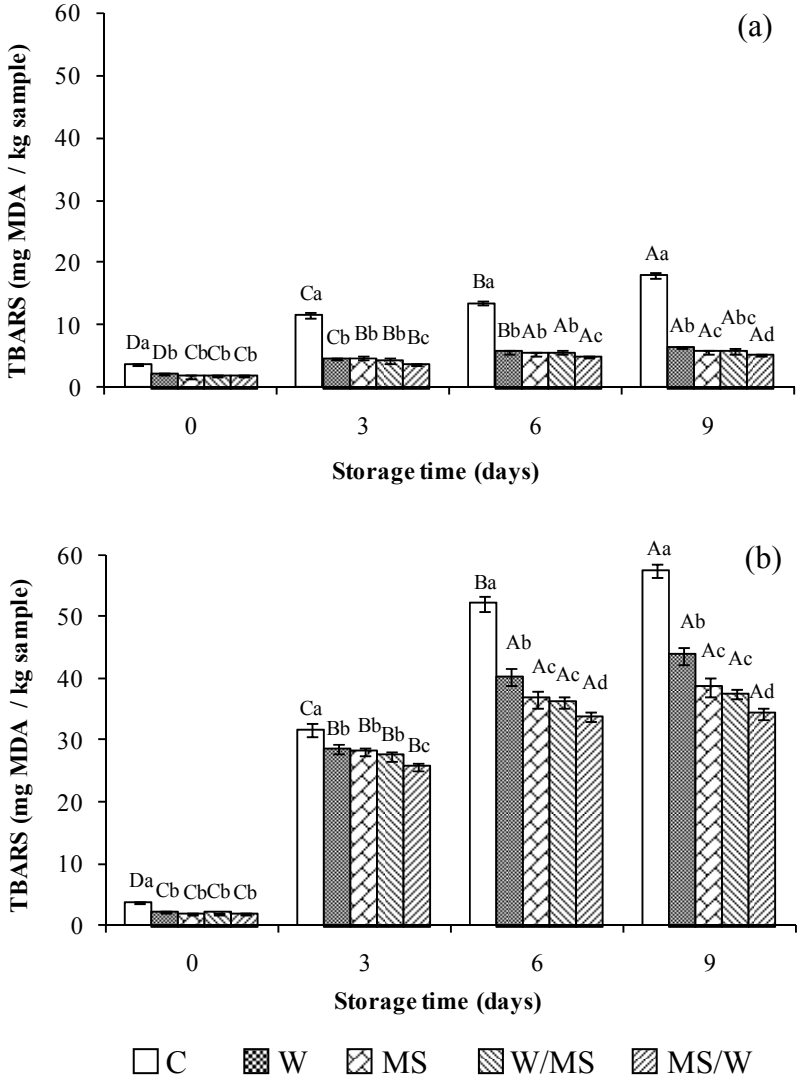

Figure 15. TBARS values of brownstripe red snapper mince with different pretreatments in the absence (a) and presence (b) of 0.15 M cupric acetate during storage at 4 °C for 9 days. C: control mince, W: washed mince, MS: membrane separated mince, W/MS: washed/membrane separated mince and MS/W: membrane separated/washed mince. Bars represent the standard deviation (n=3). Different capital letters within the same pretreatment and different letters within the same day of storage indicate significant differences ($p < 0.05$).

4.4.3 Hydrolysis of mince using protease from pyloric caeca of brownstripe red snapper

MS/W, PI and mince were used to produce protein hydrolysates using protease from pyloric caeca of brownstripe red snapper. It was noted that mince contained myosin heavy chain (MHC) and actin as the major proteins. For pretreated sample (MS/W) and PI, proteins with molecular weight of 26.9, 32.5 and 43.2 kDa disappeared (Figure 18(a)). During washing or membrane separation, some sarcoplasmic proteins and some cytoskeletal proteins interacted with phospholipid membrane were removed. Similar protein patterns were observed between MS/W and PI. For all samples, the typical initial rapid hydrolysis rate was observed within the first 30 min as evidenced by the sharp increase in DH, followed by a slower hydrolysis rate (Figure 16). Firstly, a large number of peptide bonds were hydrolyzed (Shahidi *et al*., 1995). Thereafter, proteases might have the lower activity towards the proteins or peptides with the shorter chains. At the same hydrolysis time, the higher DH was obtained in hydrolysate prepared with the higher activity of PCE ($p < 0.05$). The result suggested that higher amount of proteases used, the more peptide bonds were cleaved. At the same protease level used, PI was found to be the most susceptible to cleavage as indicated by the highest DH ($p < 0.05$).

Elimination of other proteins as well as lipids, which functioned as the barrier for hydrolysis of myofibrillar proteins by proteases, also enhanced the hydrolysis induced by proteases. Moreover, membrane separation process might cause the structural alternation of proteins by interruption of electrostatic linkage between proteins by Ca^{2+} and citric acid. Furthermore, the changes in muscle protein configuration of pretreated mince were also supported by the decreases in Mg^{2+}-ATPase activity. A decrease in Mg^{2+}-ATPase activity by 13.75 %, compared with that found in mince (data not shown), was found in natural actomyosin extracted from MS/W, indicating the disassociation of actomyosin molecule. Mg^{2+}-ATPase activity is used as an indicator of conformational changes of actomyosin complex (Benjakul *et al*., 1997). The decrease in Mg^{2+}-ATPase activity confirmed the dissociation of actin from myosin (Torigai and Konno, 1996). Therefore, the dissociation of actomyosin

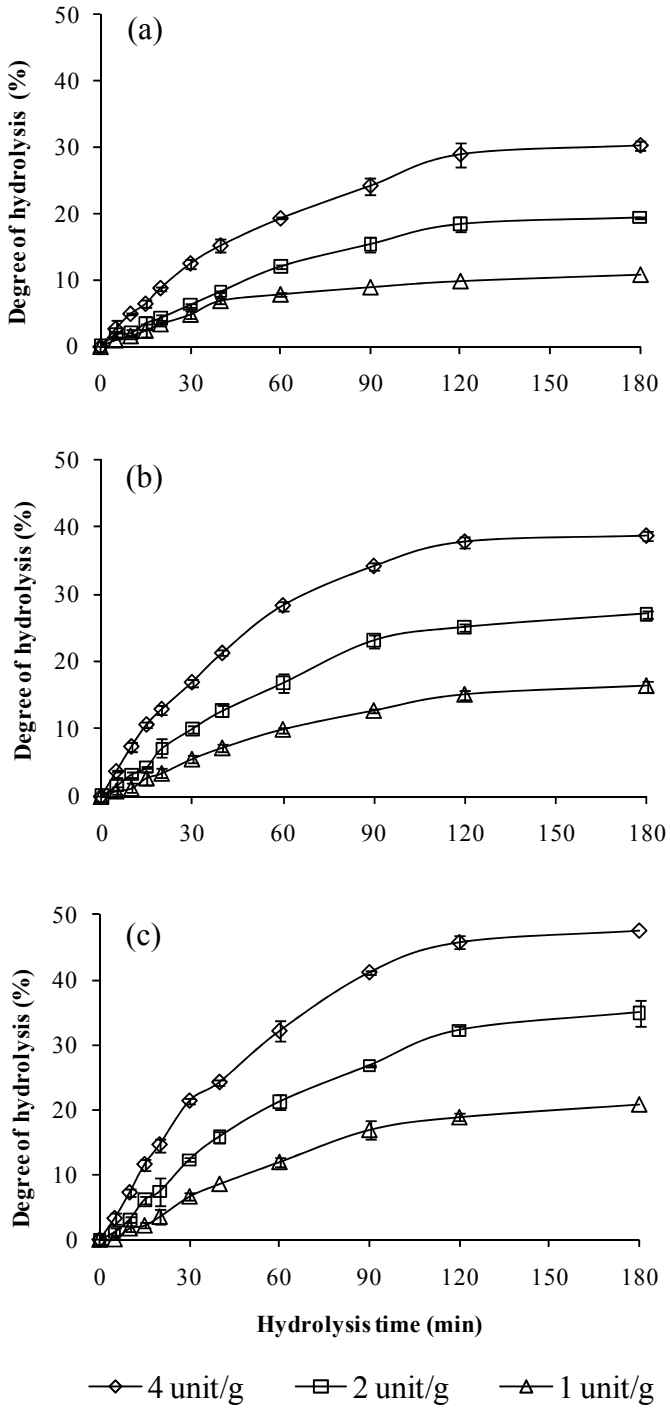

Figure 16. Changes in degree of hydrolysis of control mince (a), membrane separated/washed mince (MS/W) (b) and protein isolate (PI) (c), using protease from pyloric caeca of brownstripe red snapper at various levels. The reactions were performed at 60^{o}C and pH 8.5. Bars represent the standard deviation (n=3).

might lead to the increased susceptibility of actin and myosin to be hydrolyzed by proteases used for hydrolysis. The loosen protein structures were more prone to be cleaved by proteases. When PI was used as substrate for hydrolysis, the greater hydrolysis took place. During alkaline solubilisation, the repulsion between protein molecules resulted in the dissociation of actomyosin complex. This was indicated by the marked decrease in Mg^{2+}-ATPase activity by 36.27%, in comparison with mince (data not shown). As a consequence, the individual or looser complex could be cleaved with ease as evidenced by the higher DH. Thus, the configuration of protein was another important factor governing the rate of hydrolysis.

4.4.4 Antioxidative activities of protein hydrolysate prepared from mince with different pretreatments

4.4.4.1 DPPH radical scavenging activity

DPPH radical scavenging activities of H-C, H-MS/W and H-PI with different DHs are shown in Figure 17(a). As DH increased from 20 to 30%, H-MS/W showed the increase in DPPH radical scavenging activity but no differences were found between the samples with 30 and 40% DH ($p > 0.05$). No differences in DPPH radical scavenging activity were observed for H-C and H-PI at all DHs ($p > 0.05$). DPPH radical scavenging activity indicated the hydrogen-donating ability of antioxidant. The result suggested that H-MS/W with 30 and 40% DH could act as hydrogen donors more effectively than other hydrolysates. Protein hydrolysates from mackerel (*Scomber austriasicus*) (Wu *et al*., 2003) and round scad (*Decapterus maruadsi*) (Thiansilakul *et al.,* 2007a) prepared using enzymatic hydrolysis were reported to possess DPPH radical scavenging activity.

4.4.4.2 ABTS radical scavenging activity

H-C, H-MS/W and H-PI showed the increase in ABTS radical scavenging activities with increasing DH ($p < 0.05$) (Figure 17(b)). The highest activity was observed in all hydrolysates with 40% DH ($p < 0.05$). However, no difference in activity was found between H-PI with 20 and 30% DH ($p > 0.05$).

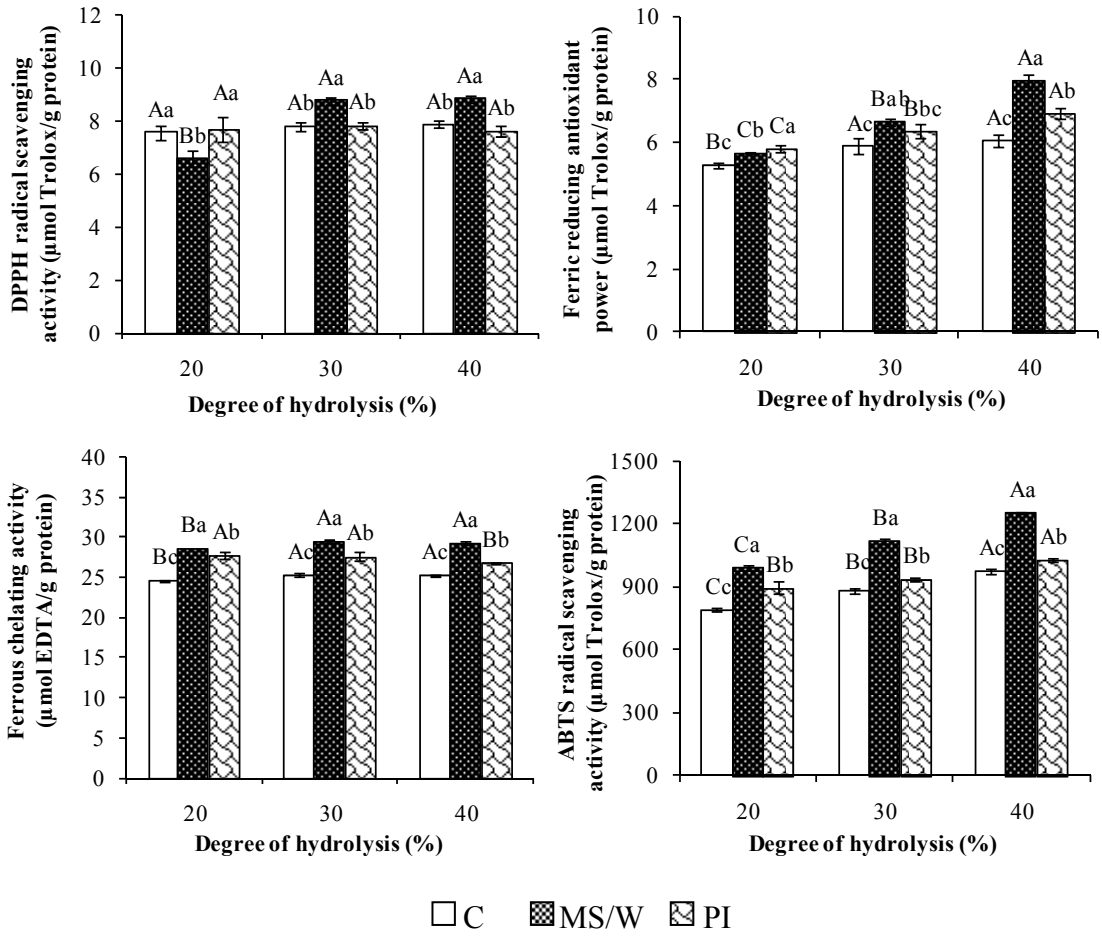

Figure 17. DPPH radical scavenging activity (a), ABTS radical scavenging activity (b), ferric reducing antioxidant power (c) and metal chelating activity (d) of hydrolysates prepared from C: control mince, MS/W: membrane separated/washed mince and PI: protein isolate, using protease from pyloric caeca with various degrees of hydrolysis (DHs). Bars represent the standard deviation (n=3). Different capital letters within the same pretreatment and different letters within the same DH indicate significant differences ($p < 0.05$).

Among the hydrolysates, H-C showed the lowest ABTS radical scavenging activity, whereas hydrolysate from MS/W exhibited the highest activity for all DH tested ($p < 0.05$). At 40% DH, H-MS/W had the highest ABTS radical scavenging activity, compared with other hydrolysates ($p < 0.05$). ABTS radical assay determine the

antioxidative activity, in which the radical is quenched to form ABTS-radical complex (Khantaphant and Benjakul, 2008) (Chapter 2). Protein hydrolysates derived from loach (You *et al*., 2009), yellow stripe trevally (Klompong *et al*., 2008) and alkali-solubilized tilapia (Raghavan *et al*., 2008) have been reported to have ABTS radical scavenging activities.

4.4.4.3 Ferric reducing antioxidant power (FRAP)

FRAP of different hydrolysates with different DH (20, 30 and 40%) is depicted in Figure 17(c). All hydrolysates had the increases in FRAP when DH increased ($p < 0.05$) except for H-C, in which no difference was observed in samples with 30 and 40% DH ($p > 0.05$). FRAP of H-MS/W and H-PI was generally higher than that of H-C at all DHs used ($p < 0.05$). At the same DH, H-MS/W showed the highest activity, in comparison with others ($p < 0.05$). FRAP generally measures the reducing ability against ferric ion (Fe^{3+}). This ability indicates the ability of hydrolysates to donor the electron to the free radical. Therefore, it could be concluded that H-MS/W, especially at high DH, had the reducing power, leading to the prevention and retardation of propagation of lipid oxidation. Hydrolysates from yellow stripe trevally (Klompong *et al*., 2008) and alkali-solubilized tilapia (Raghavan *et al*., 2008) have been reported to possess FRAP.

4.4.4.4 Ferrous chelating activity

Ferrous chelating activities of hydrolysates prepared from mince, MS/W and PI with different DHs are shown in Figure 17(d). Chelating activity against Fe^{2+} of H-C and H-MS/W slightly increased when DH increased up to 30% ($p < 0.05$). On the other hand, slight decrease in ferrous chelating activities was found in H-PI as DH was greater than 30% ($p < 0.05$). Among all hydrolysates, H-MS/W showed the highest chelating activity for all DH ($p < 0.05$). Ferrous ion (Fe^{2+}) is the most powerful pro-oxidant among metal ions. This ion can interact with hydrogen peroxide in a Fenton reaction to produce the reactive oxygen species and hydroxyl free radical (OH), leading to the initiation and/or acceleration of lipid oxidation (Stohs and Bagchi, 1995). Therefore, chelation of metal ions by peptides in hydrolysates

would retard the oxidative reaction (Klompong *et al*., 2008). The result indicated that H-MS/W was able to chelate pro-oxidative metals, resulting in the lowered oxidation. Furthermore, the higher chelating activity of H-MS/W was coincidental with the higher DPPH and ABTS radical scavenging activity and FRAP. Ferrous chelating activity has been reported for hydrolysate of silver carp (*Hypophthalmichthys molitrix*) (Dong *et al*., 2008), round scad (Thiansilakul *et al*., 2007a) and yellow stripe trevally (Klompong *et al*., 2008). Therefore, all hydrolysates could function as both primary and secondary antioxidants via scavenging free radical and chelating the metal ions, respectively. It could be observed that when mince was subjected to different pretreatments, the peptides generated showed varying activity, though the same DH was used. Therefore, different antioxidative peptides were produced from the samples with different pretreatments. Pretreatment might affect the structure of proteins, in which hydrolysis could take place at different positions in peptide chains. Thiansilakul *et al*. (2007a) and Klompong *et al*. (2008) reported that the differences in antioxidative activities among hydrolysates might result from the differences in the structure and size of the peptides of the resulting hydrolysate. Since the hydrolysis was performed using the same protease, the different antioxidative activities of resulting hydrolysate was governed by pretreatments. DH also greatly influenced the peptide chain length as well as the exposure of terminal amino groups of products obtained (Thiansilakul *et al*., 2007b). The higher DH, the more cleavage of peptide chains took place. Peptides with various sizes and compositions had various capacities of scavenging or quenching free radicals (Klompong *et al*., 2007).

4.4.5 Protein pattern of protein hydrolysate prepared from mince with different pretreatments

Protein patterns of hydrolysates from mince without and with pretreatment having 40% DH are shown in Figure 18(b). For mince with different pretreatments, myosin heavy chain (MHC) and actin were the major components. Slight decrease in MHC band intensity was noticeable in MS/W and PI, probably due to slight degradation of MHC during the process. After hydrolysis, no MHC and actin

were detectable. H-C showed two major protein bands with molecular weight (MW) of 5.8 and 10.4 kDa. H-MS/W and H-PI showed smear bands with MW lower than 6.5 kDa. However, the smear band of H-MS/W was found with MW range of 5.1-6.5 kDa and protein bands with MW of 4.6-10.4 kDa were found in H-PI. The result indicated that the types and sizes formation of peptides in hydrolysate from mince with different pretreatments varied. In general, the hydrolysates prepared at 40% DH showed the highest antioxidative activities. Therefore, hydrolysates with 40% DH were selected to study for their ability in inhibition of lipid oxidation in lecithin-liposome oxidation model system, in comparison with commercial antioxidants.

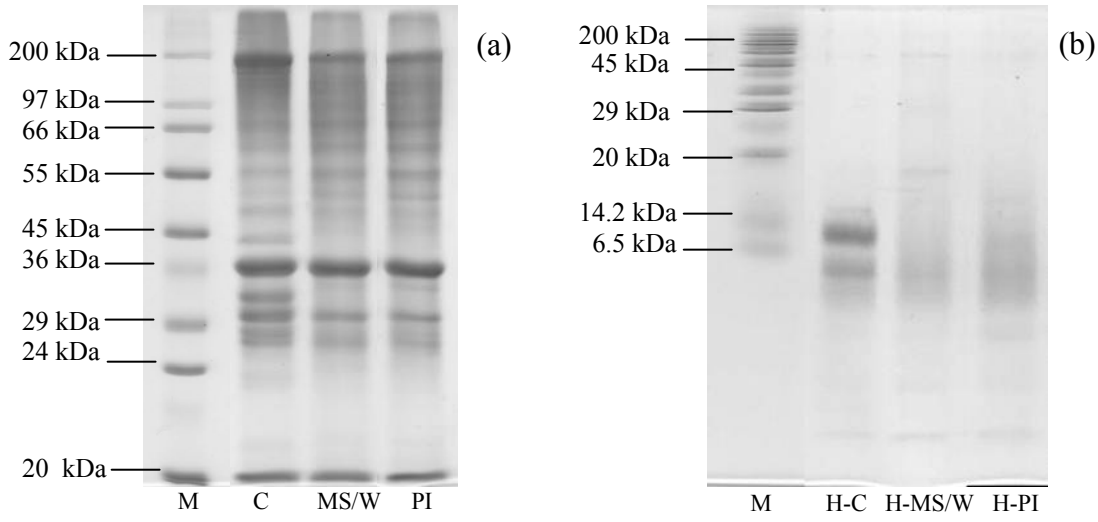

Figure 18. Protein pattern of brownstripe red snapper mince with different pretreatments before (a) and after (b) hydrolysis with protease from pyloric caeca to obtain 40% DH. M: molecular weight marker; C: control mince; MS/W: membrane separated/washed mince, PI: protein isolate, H-C hydrolysate from C, H-MS/W: hydrolysate from MS/W and H-PI: hydrolysate from PI.

4.4.6 Antioxidative activity of hydrolysate from mince with different pretreatments in a lecithin liposome system

Protein hydrolysates prepared from mince with different pretreatments affected the oxidation of the lecithin liposome system differently as indicated by different conjugated dienes and TBARS values (Figure 19(a) and (b)), respectively. No changes in conjugated dienes of liposome system added with 100 ppm BHT was observed throughout 48 h of incubation ($p > 0.05$). For the system added with 25 ppm BHT, the increase in conjugated dienes was found after 12 h of incubation. An increase in conjugated dienes followed by a sharp decrease was observed from the control system and all systems containing all hydrolysates at a level of 1000 ppm but the rate of changes varied with the hydrolysate added (Figure 19(a)). Within the first 12 h of incubation, liposome system added with H-C and the control system showed the higher conjugated diene than those added with H-MS/W, H-PI and BHT ($p < 0.05$). The results indicated that H-MS/W and H-PI in liposome system could retard the formation of conjugated diene. The higher conjugated diene in the system added with H-C might be due to the remaining pro-oxidants in minces, which could induce the oxidation within the first period of incubation. However, after 12 h, the sharp decreases in conjugated diene were observed from the system added with H-C and the control, indicating the transformation of conjugated diene into hydroperoxide induced by oxidation process. The lower conjugated diene content was associated with the higher formation of hydroperoxide in propagation stage. Conjugated diene is generated during the early stage of lipid oxidative reaction and hydroperoxides are expected to decompose to the secondary products which can be determined by TBARS value (Frankel *et al*., 1997).

Changes in TBARS of liposome system during incubation of 48 h are shown in Figure 19 (b). The increase in TBARS indicated the formation of secondary lipid oxidation products. The sharp increases in TBARS were observed in the first 18 h of incubation ($p < 0.05$), when H-C was added to liposome system. The systems added with H-MS/W and H-PI showed the increase in TBARS within the first 24 h ($p < 0.05$). Thereafter, the gradual decreases in TBARS were observed ($p < 0.05$).

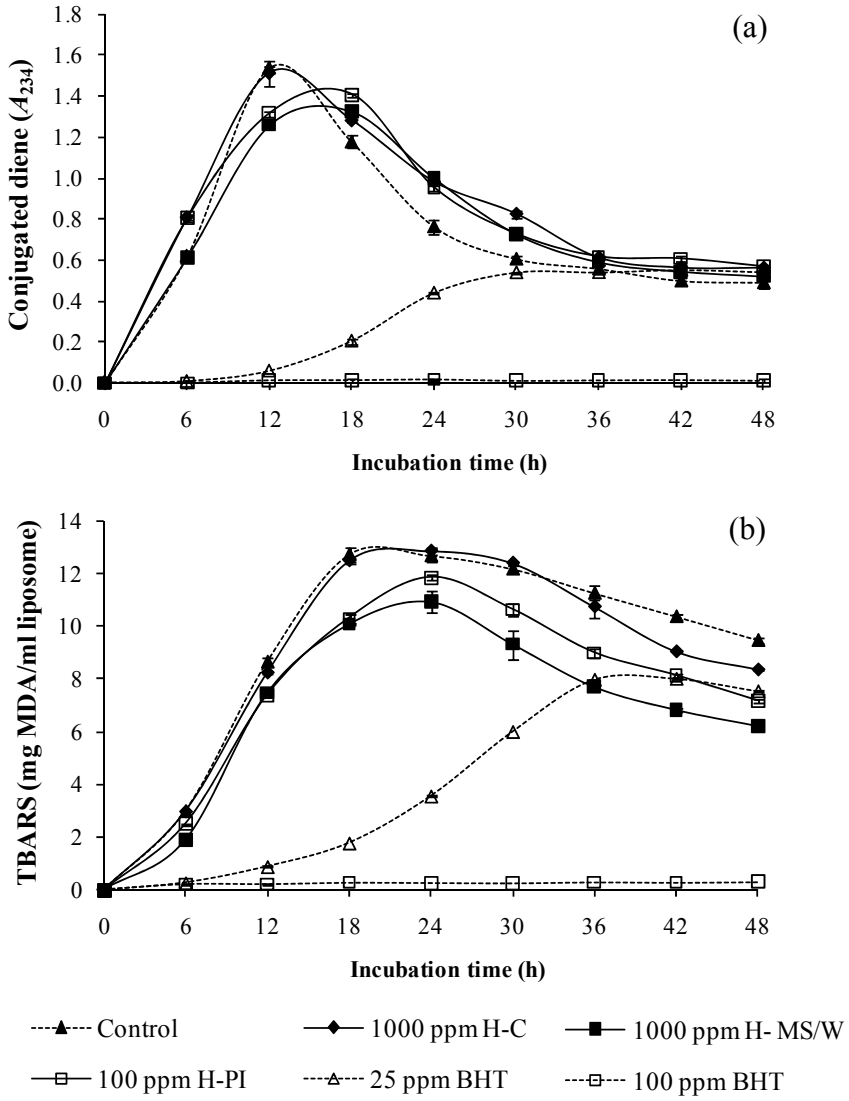

Figure 19. The formation of conjugated diene (a) and thiobarbituric acid reactive substances (TBARS) (b) in lecithin-liposome model system in the presence of different hydrolysates from brownstripe red snapper mince with different pretreatments having 40% DH. Bars represent the standard deviation (n=3).

However, the value of TBARS obtained from the former system was lower than that of the latter ($p < 0.05$). A gradual decrease in TBARS was found during 18 - 48 h for the control and the system added with H-C and during 24 to 48 h for the systems added with H-MS/W and H-PI (Figure 19 (b)). The result suggested the greater ability

of H-MS/W and H-PI in retardation of the formation of secondary oxidation products determined by TBARS. The first formation of TBARS indicated that the primary products were decomposed to the secondary products, especially aldehydes. The decrease in TBARS when the storage time increased was probably due to the loss in those volatile secondary products. In general, the increases in conjugated diene and TBARS values of the liposome systems containing H-MS/W and H-PI were lower than that of the control and the system containing H-C. Thus, H-MS/W and H-PI could retard the early stages of lipid oxidation (formation of conjugated diene) as well as the advanced stage of oxidation via their radical scavenging activity, reducing power and metal chelating activity. Liposomes are appropriate lipid models to evaluate antioxidants for both food and lipoprotein particles containing phospholipids (Frankel *et al*., 1997). Polar portions of peptides in hydrolysate might interact with the liposomes of phospholipids, where they functioned effectively as antioxidant at the interface.

4.5 Conclusion

Pretreatment of mince played a role in lowering pro-oxidants and phospholipid membrane in mince. Cleavage of protein was affected by pretreatment and antioxidative activity of protein hydrolysate was also governed by pretreatment. Fish mince subjected to membrane separation, followed by washing was appropriate for production of hydrolysate possessing antioxidative activity with the lesser amount of pro-oxidants and phospholipid membrane, which can be further used to retard lipid oxidation in food system.

CHAPTER 5

ANTIOXIDATIVE AND ACE INHIBITORY ACTIVITIES OF PROTEIN HYDROLYSATES FROM THE MUSCLE OF BROWNSTRIPE RED SNAPPER PREPARED USING PYLORIC CAECA AND COMMERCIAL PROTEASES

5.1 Abstract

Protein hydrolysates from the muscle of brownstripe red snapper (*Lutjanus vitta*) prepared using Alcalase or Flavourzyme as the first step with 40% degree of hydrolysis (DH), followed by hydrolysis with pyloric caeca protease (PCP) as the second step for 2 (HAP) and 1 h (HFP), respectively, were prepared and determined for their antioxidative and angiotensin I-converting enzyme (ACE) inhibitory activities. HAP exhibited the higher DPPH and ABTS radical scavenging activity and ferric reducing antioxidant power (FRAP), while HFP showed the higher ferrous chelating activity and ACE inhibitory activity ($p < 0.05$). Both HAP and HFP were able to retard lipid oxidations in lecithin-liposome, β-carotene-linoleic acid and comminuted fish model systems in dose-dependent manner. HFP showed a slightly greater efficiency in prevention of lipid oxidation in all systems tested. Antioxidative activities, except DPPH radical scavenging activity, in gastrointestinal tract model system (GIMs) of both HAP and HFP increased, indicating the enhancement of antioxidative activities after ingestion.

5.2 Introduction

Lipid oxidation is one of the major deterioration in many types of natural and processed foods, leading to the changes in food quality and nutritional value. Additionally, potentially toxic reaction products can be produced. Lipid oxidations have been known to be the major causes of many serious human diseases, such as cardiovascular disease, cancer, and neurological disorders as well as the aging process (Jittrepotch *et al*., 2006). To prevent oxidative deterioration of foods and to provide protection against serious diseases, it is important to inhibit the oxidation of lipids and the formation of free radicals occurring in the foodstuff and living body. To tackle the problem, antioxidants, both natural and synthetic ones, have been used widely. Nevertheless, synthetic antioxidants have been suspected of being responsible for toxicity in the long term and their use in foodstuffs is restricted or prohibited in some countries (Sakanaka *et al*., 2004; Je *et al*., 2005; Pihlanto *et al*., 2008). Therefore, there is a growing interest on natural antioxidants, especially peptides derived from hydrolyzed food proteins (Gulcin, 2006). Protein hydrolysate from several fish species including round scad (*Decapterus maruadsi*) (Thiansilakul *et al*., 2007a), yellow stripe trevally (*Selaroides leptolepis*) (Klompong *et al*., 2007), Pacific hake (*Merluccius productus*) (Samaranayaka and Li-Chan 2008), tilapia (*Oreochromis niloticus*) (Raghavan *et al*., 2008), silver carp (*Hypophthalmichthys molitrix*) (Dong *et al*., 2008) and smooth hound (*Mustelus mustelus*) (Bougatef *et al*., 2009) have been reported to possess antioxidative activities.

Hypertension has been considered the most common serious chronic health problem (Jung *et al*., 2006; You *et al*., 2009). Since angiotensin I-converting enzyme (ACE) (EC 3.4.15.1) is physiologically important in raising blood pressure, the inhibition of ACE activity can lead to an overall antihypertensive effect. The synthetic ACE inhibitors are now widely used as pro-drugs but these synthetic ACE inhibitors can cause many significant undesirable side effects (Ono *et al*., 2006). Therefore, the natural safe compounds are desirable for prevention of hypertension instead of the synthetic counterpart. Among those, food protein derived peptides are the promising natural products exhibiting ACE inhibitory activities (Jeon *et al*., 1999).

Protein hydrolysates with antihypertensive activity were also produced from sardinelle (*Sardina pilchardus*) by-products (Bougatef, *et al*., 2008), tuna cooking juice (Ko *et al*., 2006), salmon protein (Ewart *et al*., 2009), chum salmon (*Oncorhynchus keta*) (Ono *et al*., 2003; 2006) and tilapia (Raghavan and Kristinsson, 2009).

In Thailand, brownstripe red snapper (*Lutjanus vitta*) is one of the raw materials for surimi production (Khantaphant and Benjakul, 2008) (Chapter 2). Besides being produced into surimi, this species and its viscera, especially pyloric caeca, can be used as raw material for production of protein hydrolysate and as the source of proteases, respectively. Production of fish protein hydrolysates with bioactivity can pave the way for full utilization of these species. Many factors affecting the bioactivity of protein hydrolysates, e.g. type of proteases (Klompong *et al*., 2007), steps of hydrolysis (Phanturat *et al*., 2010), etc. This work aimed to study antioxidative and ACE inhibitory activities of protein hydrolysate from brownstripe red snapper muscle prepared using its pyloric caeca protease in combination with commercial proteases via 2-step hydrolysis and to investigate the bioactivities of selected hydrolysate after digestion in gastrointestinal tract model system.

5.3 Materials and Methods

5.3.1 Chemicals

Alcalase 2.4 L (E.C 3.4.21.62) (2.4 AU/g) and Flavourzyme 500 L (E.C 3.4.21.77) (500 LAPU/g) were provided by Novozyme (Bagsvaerd, Denmark). 2,2′-azinobis (3-ethylbenzothiazoline-6-sulfonic acid) (ABTS), 1,1-diphenyl-2-picrylhydrazyl (DPPH), 1,1,3,3-tetramethoxypropane, 3-(2-pyridyl)-5,6-diphenyl-1,2,4-triazine-4′,4″-disulfonic acid (ferrozine), 2, 4, 6-tripyridyl-triazine (TPTZ), *L*-*α*-phosphatidylcholine (lecithin) and linoleic acid were purchased from Sigma (St. Louis, MO, USA). Thiobarbituric acid (TBA), potassium persulfate, *β*-carotene and Tween 40 were obtained from Fluka (Buchs, Switzerland) and sodium sulfite was

obtained from Riedel-deHaën (Seelze, Germany). All chemicals were of analytical grade.

5.3.2 Preparation of pretreated fish mince

Brownstripe red snapper, off-loaded approximately 24-36 h after capture, were purchased from a dock in Songkhla province, Thailand. Fish were transported in ice with the fish/ice ratio of 1:2 (w/w) to the Department of Food Technology, Prince of Songkla University within 1 h. Upon arrival, whole fish were washed and only flesh was separated manually. Flesh was minced to uniformity using Moulinex AY46 blender (Group SEB, Lyon, France) before further homogenizing with nine volumes of cold 8 mM $CaCl_2$ solution containing 5 mM citric acid using an IKA Labortechnik homogenizer (Selangor, Malaysia) at a speed of 11,000 rpm for 2 min. After continuous stirring for 60 min at 4°C, the sample was centrifuged at 4,000 x *g* for 15 min at 4°C using a Beckman Coulter centrifuge Model Avant J-E (Beckman Coulter, Inc., Fullerton, CA, USA). Thereafter, the pellet was washed by homogenizing with five volumes of cold distilled water using a homogenizer at a speed of 11,000 rpm for 2 min, followed by stirring at 4°C for 15 min prior to centrifuging at 9,600 x *g* for 10 min at 4°C. The washing process was repeated twice. Pretreated mince obtained was kept in polyethylene bags and placed in ice until use, but not longer than 2 h.

5.3.3 Preparation of proteases from pyloric caeca

Pyloric caeca from brownstripe red snapper was collected and powdered in liquid nitrogen. Thereafter, the pyloric caeca extract was prepared according to the method of Khantaphant and Benjakul (2010) (Chapter 3). Pyloric caeca powder was suspended in ten volumes of 50 mM Tris-HCl buffer, pH 8.0, containing 10 mM $CaCl_2$. The mixture was homogenized at 11,000 rpm for 2 min. The homogenate was continuously stirred for 30 min at 4°C and centrifuged at 8,000 x *g* for 30 min at 4°C. The supernatant was filtered through a Whatman filter paper

No. 1 (Whatman International Ltd., Maidstone, England). The filtrate obtained was further subjected to 40-60% saturation ammonium sulfate precipitation. After stirring at 4°C for 30 min, the mixture was centrifuged at 8,000 x *g* for 30 min at 4°C and the pellet obtained was dissolved in 50 mM Tris-HCl buffer, pH 8.0 followed by dialysis 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 'pyloric caeca protease; PCP'. PCP was determined for proteolytic activity using casein as a substrate under optimal condition (60°C and pH 8.5).

5.3.4 Preparation of protein hydrolysate from brownstripe red snapper

5.3.4.1 One-step hydrolysis using different single proteases

Pretreated brownstripe red snapper mince with a protein content of 95.42% protein (dry basis) determined by Kjeldahl method (AOAC, 2000) was mixed with 50 mM Tris-HCl buffer with pH of 7.0, 8.0 and 8.5 for hydrolysis by Flavourzyme, Alcalase and PCP, respectively, to obtain a final protein concentration of 2% (w/v). The mixtures were homogenized at a speed of 11,000 rpm for 1 min and the homogenates were pre-incubated for 10 min at 50°C for Alcalase and Flavourzyme and at 60°C for PCP. To start the hydrolysis, the different levels of enzymes were added into the mixture to obtain the desirable degree of hydrolysis (DH) of 20, 30 and 40% following the method of Benjakul and Morrissey (1997). After 2 h of hydrolysis, the reactions were inactivated by placing the mixture in boiling water for 10 min. Thereafter, the mixture was centrifuged at 2,000 x *g* at 4°C for 10 min. The supernatant was collected and referred to as protein hydrolysate prepared using Alcalase (HA), Flavourzyme (HF) or PCP (HP). All protein hydrolysates were determined for antioxidative activities.

5.3.4.2 Two-step hydrolysis using different proteases

After the first hydrolysis for 2 h to obtain the DH of 40%, which exhibited the highest antioxidative activities, the mixtures were heated for 10 min in boiling water and adjusted to the desirable pH using 1 N NaOH or 1 N HCl for

proteases used for the second step. Those proteases included Alcalase, Flavourzyme and PCP. The same amount of proteases used in the first step was added into the pre-incubated mixture with optimal temperature of each protease. Reaction was conducted for 1, 2, 3 and 5 h. At the time designated, the reaction was terminated by submerging the mixture in boiling water for 10 min. The mixture was then centrifuged at 2,000 x *g* at 4°C for 10 min and the supernatant was collected and adjusted to neutral pH. The neutral solution was referred to as 'protein hydrolysates' and lyophilized to obtain hydrolysate powder. The hydrolysates obtained from HP with further hydrolysis using Alcalase (HPA) or Flavourzyme (HPF), hydrolysate from HA using PCP or Flavourzyme for the second step (HAP and HAF) and hydrolysate from HF using PCP or Alcalase for the second step (HFP and HFA) were determined for their antioxidative and ACE inhibitory activities.

5.3.5 Determination of antioxidative activities

5.3.5.1 DPPH radical scavenging activity

DPPH radical scavenging activity was determined according to the method of Khantaphant and Benjakul (2008) (Chapter 2). Sample solution (1.5 mL) was added with 1.5 mL of 0.1 mM DPPH in 95% ethanol. The mixture was allowed to stand for 30 min in dark at room temperature. The resulting solution was measured at 517 nm. The control was prepared in the same manner except that distilled water was used instead of the sample. The DPPH radical scavenging activity was calculated from Trolox standard curve (0-60 μM) and expressed as μmol Trolox equivalents (TE)/g protein.

5.3.5.2 ABTS radical scavenging activity

ABTS radical scavenging activity was determined as described by Re *et al*. (1999). ABTS radical ($ABTS^{\bullet+}$) was produced by reacting ABTS stock solution with 2.6 mM potassium persulfate at the ratio of 1:1 (v/v). The mixture was allowed to react in dark for 12 h at room temperature. Prior to assay, $ABTS^{\bullet+}$ solution was diluted with methanol to obtain an absorbance of 1.1 (±0.02) at 734 nm. To initiate

the reaction, 150 µL of sample was mixed with 2.85 mL of $ABTS^{\bullet+}$ solution. The mixture was incubated at room temperature for 2 h in dark. The absorbance was then read at 734 nm. Trolox standard curve (0-200 µM) was prepared. Distilled water was used instead of the sample and prepared in the same manner to obtain the control. ABTS radical scavenging activity was expressed as µmol TE/g protein.

5.3.5.3 Ferric reducing antioxidant power (FRAP)

The ability of samples to reduce ferric ion (Fe^{3+}) was evaluated by the method of Benzie and Strain (1996). FRAP reagent (a freshly prepared mixture of 10 mM TPTZ solution in 40 mM HCl, 20 mM $FeCl_3.6H_2O$ solution and 300 mM acetate buffer, pH 3.6 (1:1:10 v/v/v)) (2.85 mL) was incubated at 37°C for 30 min prior to mixing with 150 µL of sample. The reaction mixture was allowed to stand in dark for 30 min at room temperature. Absorbance at 593 nm was read and FRAP was calculated from the Trolox standard curve (0-60 µM) and expressed as µmol TE/g protein. The control was prepared in the same manner except that distilled water was used instead of the sample.

5.3.5.4 Ferrous chelating activity

Chelating activity of samples towards ferrous ion (Fe^{2+}) was measured by the method of Benjakul *et al*. (2005a) with slight modification. Sample (200 µL) was mixed with 800 µL of distilled water. Thereafter, 0.1 mL of 2 mM $FeCl_2$ and 0.2 mL of 5 mM ferrozine were added. The mixture was allowed to react for 20 min at room temperature. The absorbance was then read at 562 nm. The standard curve of EDTA (0-1.0 mM) was prepared. The control was prepared in the same manner except that distilled water was used instead of the sample. Ferrous chelating activity was expressed as µmol EDTA equivalents/g protein.

5.3.6 Determination of ACE inhibitory activity

The angiotensin I-converting enzyme inhibitory activity was determined as described by Hayakari *et al*. (1978) with slight modifications. Sample

(0.3 mL) was incubated with 0.725 unit ACE/mL (50 µL) at 37°C for 5 min. Thereafter, the incubated mixture was added into the assay mixture (100 mM K_2HPO_4 buffer (pH 8.3) containing 600 mM NaCl and 3 mM hippuryl-L-histidyl-L-leucine (HHL))(0.2 mL). The mixture was incubated at 37°C for 15 min. To terminate the reaction, 1.5 mL of 3% 2,4,6-trichloro-1,3,5-triazine (dissolved in dioxane) and 3 mL of 0.2 M K_2HPO_4 buffer (pH 8.3) were added and mixed thoroughly. The mixture was left for 10 min until the solution was clear and the absorbance at 382 nm was determined. Sample blank were prepared in the same manner except that HHL was added after the reaction mixture was terminated. The control was prepared by using distilled water instead of the sample, whereas the control blank was done in the same manner with the control but HHL was added after the termination. % inhibition of ACE was determined using following equation:

$$\% \text{ Inhibition} = (A_C - A_S) / A_C \times 100$$

where $A_C = A_{\text{control}} - A_{\text{control blank}}$ and $A_S = A_{\text{sample}} - A_{\text{sample blank}}$

5.3.7 Determination of antioxidative activity in different oxidation model systems

5.3.7.1 *β*-carotene linoleic acid emulsion model system

The antioxidative activity of the sample in the *β*-carotene linoleic acid emulsion model system was determined as described by Binsan *et al*. (2008). *β*-carotene (1 mg) was dissolved in 10 mL of chloroform. Thereafter, the solution (3 mL) was added to 20 mg linoleic acid and 200 mg Tween 40. Chloroform was then removed by purging with nitrogen. Fifty mL of oxygenated distilled water were added to the *β*-carotene emulsion and mixed well. Hydrolysate (200 µL) was then mixed with 3 mL of oxygenated *β*-carotene emulsion to obtain the final concentrations of 500 and 1,000 ppm. The oxidation of *β*-carotene emulsion was monitored spectrophotometrically at 470 nm after 0, 10, 20, 30 40, 60, 90 and 120 min of

incubation at 50°C in dark. BHT at levels of 100 ppm was also used. The control was prepared by using distilled water instead of hydrolysate in the assay system.

5.3.7.2 Lecithin liposome model system

The antioxidative activity of protein hydrolysates in a lecithin liposome system was determined according to the method of Frankel *et al*. (1997) with slightly modified by Thiansilakul *et al*. (2007a). Lecithin liposome system was prepared by suspending lecithin in deionized water at a concentration of 8 mg/mL. The mixture was stirred with a glass rod followed by sonicating for 30 min in a sonicating bath (Elma Model S30H, Singen, Germany). Protein hydrolysate (3 mL) was added to the lecithin liposome system (15 mL) to obtain a final concentration of 1,000 ppm. 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 at 120 rpm using a shaker (Heidolph Model Unimax 1010, Schwabach, Germany) at 37°C in dark. The systems containing 25 or 100 ppm BHT were also prepared. The control was prepared in the same manner, except that distilled water was used instead of sample. Oxidation in lecithin liposome systems was monitored at 6 h intervals by determining the formation of TBARS and conjugated dienes.

5.3.7.3 Comminuted fish model system

The comminuted fish model system was prepared according to the method of Benjakul *et al*. (2005a) with a slight modification. Round scads (*Decapterus maruadsi*), a dark fleshed fish, and brownstripe red snapper, a lean fish, were washed, filleted and minced. The mince (100 g) was mixed well with 20 mL of distilled water (w/w) and used as the control. The hydrolysate (20 mL) was added into the fish mince (100 g) to obtain the final concentrations of 1,000 ppm. BHT (200 ppm) was also used. All mixtures were then mixed with sodium azide to obtain a final concentration of 0.01% (w/w). The mixtures were placed in polyethylene bags and kept at 4°C. After the designated storage time (0, 2, 4, 6, 8 and 10 days), all samples were randomly taken for TBARS determination.

5.3.8 Determination of thiobarbituric acid reactive substances (TBARS)

Thiobarbituric acid reactive substances (TBARS) were determined as described by Benjakul *et al*. (2005) with a slight modification. Sample was homogenized with TBARS solution (0.375% TBA, 15% TCA and 0.25 M HCl) with a ratio of 1:4 (w/v). 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 x *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 UV-1800 Spectrophotometer (Shimadzu, Kyoto, Japan). TBARS was calculated from a standard curve of malonaldehyde (MDA) and expressed as mg MDA/kg sample.

5.3.9 Determination of conjugated diene

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

5.3.10 Determination of protein concentration

Protein concentration was measured by the method of Lowry *et al*. (1951) using bovine serum albumin as a standard.

5.3.11 Preparation of gastrointestinal tract model system (GIMs)

Gastrointestinal tract model system was prepared according to the method of Lo *et al*. (2006) with slight modification. Hydrolysate powder was dissolved in distilled water to obtain the concentration of 5% (w/v) protein. The solution was adjusted to pH 2.0 with 1 N HCl and pepsin dissolved in 0.1 M HCL was added to obtain the final concentration of 4% (w/w protein). The mixture was

incubated at 37 °C for 1 h with continuous shaking (Memmert Model SV 1422, Schwabach, Germany). Thereafter, the pH of reaction mixture was raised to 5.3 with 1 N NaOH before adding 2% pancreatin (w/w of protein) and the pH of mixture was adjusted to 7.5 with 1 N NaOH. The mixture was incubated at 37 °C for 3 h with continuous shaking. During digestion, the mixture was randomly taken and determined for antioxidative activities at 0, 20, 40, 60, 80, 100, 120, 150, 180, 210 and 240 min. The digestion was terminated by submerging the mixture in boiling water for 10 min.

5.3.12 Statistical analysis

Experiments were run in triplicate. All data were subjected to analysis of variance (ANOVA) and differences between means were evaluated by Duncan's Multiple Range Test (Steel and Torrie, 1980). SPSS Statistic Program (Version 10.0) (SPSS Inc, Chicago, IL, USA) was used for data analysis.

5.4 Results and Discussion

5.4.1 Antioxidative activities of brownstripe red snapper protein hydrolysates prepared with single step of hydrolysis using different proteases

Protein hydrolysates from brownstripe red snapper muscle prepared using protease from pyloric caeca of brownstripe red snapper, Alcalase and Flavourzyme referred to as HP, HA and HF, respectively, with different DH (20, 30 and 40% DH) showed the varying antioxidative activities (Figure 20).

5.4.1.1 DPPH radical scavenging activity

All protein hydrolysates prepared using various enzymes had the increases in DPPH radical scavenging activity as DH increased ($p < 0.05$) (Figure 20(a)), except for HF which showed the similar activity at all DH used ($p > 0.05$). At all designated DH, HA and HF showed the higher activity than did HP ($p < 0.05$). The

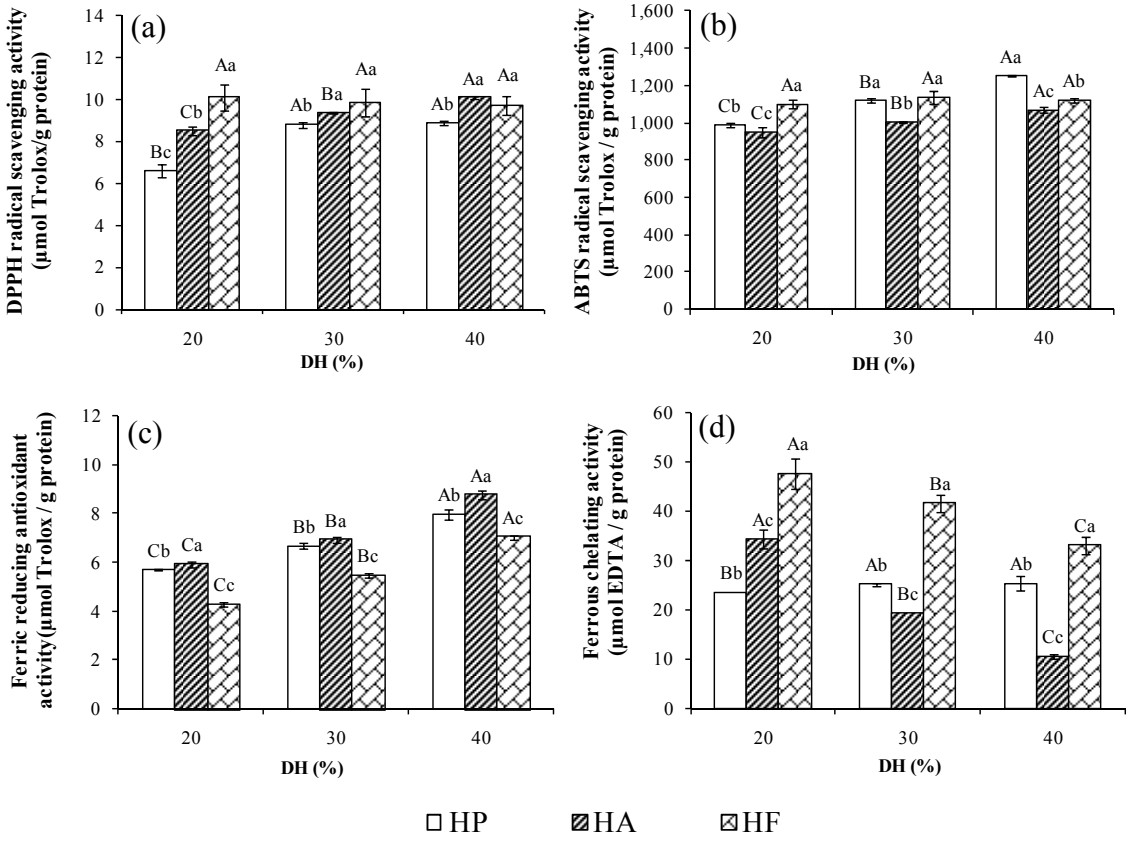

Figure 20. Antioxidative activities of protein hydrolysate from brownstripe red snapper muscle prepared using pyloric caeca protease (PCP) from brownstripe red snapper, Alcalase or Flavourzyme with different DHs. HP, HA and HF: hydrolysate prepared using PCP, Alcalase and Flavourzyme, respectively. Bars represent standard deviation (n=3). Different capital letters within the same enzyme used and different letters within the same DH indicate significant differences ($p < 0.05$).

result suggested that the peptides in different hydrolysates might be different in term of chain length and amino acid sequence, which contributed to varying capability of scavenging DPPH radicals. The result was in agreement with Thiansilakul *et al*. (2007a) who reported that the increases in DPPH radical scavenging activity of the hydrolysate from round scad muscle protein prepared using Flavourzyme and Alcalase as DH increased. On the other hand, Klompong *et al*. (2007) found that

DPPH radical scavenging activity of protein hydrolysate prepared from the muscle of yellow stripe trevally using Flavourzyme and Alcalase decreased when DH increased. Invert correlation between DH and DPPH radical scavenging activity was obtained for protein hydrolysates prepared from alkaline-aided channel catfish protein isolates using Protamex (Theodore *et al*., 2008). You *et al*. (2009) reported that loach protein hydrolysate showed the greater DPPH radical scavenging activity when DH increased. DPPH is a stable free radical and can be scavenged with a proton-donating substance, such as an antioxidant (Khantaphant and Benjakul, 2008) (Chapter 2). Therefore, protein hydrolysates from brownstripe red snapper muscle more likely contained peptides acting as hydrogen donors, thereby scavenging free radicals by converting them into more stable products.

5.4.1.2 ABTS radical scavenging activity

In general, ABTS radical scavenging activities of protein hydrolysates increased as DH increased ($p < 0.05$) (Figure 20(b)). The highest activity was observed in HP and HA with 40% DH ($p < 0.05$). However, no difference in activity was found in HF with all DH used ($p > 0.05$). Among all hydrolysates, HA exhibited the lowest activity for all DH tested ($p < 0.05$). At DH of 20%, HF showed the higher ABTS radical scavenging activity than did HP ($p < 0.05$). Conversely, HP had the highest activity when DH of 40% was used ($p < 0.05$). Protein hydrolysate from alkali-solubilized tilapia protein prepared using various proteases showed the sharp increase in ABTS radical scavenging activity when DH increased (Raghavan *et al*., 2008). Loach protein hydrolysate showed the similar result, in which ABTS radical scavenging activity increased with increasing DH (You *et al*., 2009).

For both DPPH and ABTS assays, HF showed no differences in activities with all DH tested ($p > 0.05$). ABTS radical assay is an excellent tool for determining the antioxidative activity, in which the radical is quenched to form ABTS-radical complex. Generally, all hydrolysates contained peptides, which were able to scavenge ABTS radicals, leading to the termination of radical chain reaction.

5.4.1.3 Ferric reducing antioxidant power (FRAP)

Ferric reducing antioxidant power (FRAP) generally measures the reducing ability against ferric ion (Fe^{3+}), indicating the ability of hydrolysates to transfer an electron to the free radical. FRAP of different hydrolysates with varying DH is depicted in Figure 20(c). All hydrolysates had the increases in FRAP when DH increased ($p < 0.05$). FRAP of HA was generally higher than those of HP and HF at all DHs tested ($p < 0.05$). The result suggested that HA had the greater reducing power than did others, leading to the greater efficacy in prevention and retardation of propagation in lipid oxidation. However, protein hydrolysates prepared from alkaline-aided channel catfish protein isolates had the decrease in reducing power with increasing DH (Theodore *et al*., 2008). Raghavan *et al*. (2008) reported that alkaline-solubilized tilapia protein hydrolysate prepared using Flavourzyme showed the increase in reducing ferric ion when DH increased. The hydrolysis most likely increased the reducing power, especially when the cleavage of peptides increased as indicated by the increase in DH. The result indicated that peptides generated from the hydrolysis by different proteases exhibited the different capacities of providing electron to the radicals.

5.4.1.4 Ferrous chelating activity

Ferrous ion (Fe^{2+}) is the most powerful pro-oxidant among metal ions (Yomauchi *et al*., 1988), leading to the initiation and acceleration of lipid oxidation by interaction with hydrogen peroxide in a Fenton reaction to produce the reactive oxygen species, hydroxyl free radical (OH˚) (Hultin, 1994). Therefore chelation of metal ions by peptides in hydrolysates would retard the oxidative reaction. Ferrous chelating activities of hydrolysates prepared using different proteases with different DHs are shown in Figure 20(d). Chelating activity against Fe^{2+} of HP slightly increased when DH increased up to 30% ($p < 0.05$). For HA and HF, the decreases in ferrous chelating activities were found as DH increased ($p < 0.05$). The result indicated that a higher DH rendered HA and HF with lower metal-chelating activities. The shorter chain of peptides might lose their ability to form the complex with Fe^{2+}. At DH of 20%, HF showed the highest chelating activity ($p < 0.05$), followed by HA

and HP, respectively. Peptides in HF could effectively chelate the Fe^{2+}, leading to the retardation of initiation stage. The result indicated that the limited hydrolysis of muscle protein resulted in the enhanced ferrous chelating activity, compared with the excessive hydrolysis. It was noted that the higher chelating activity of HP was coincidental with the higher DPPH and ABTS radical scavenging activity and FRAP, as the DH increased. Fe^{2+} chelating activity of round scad protein hydrolysate prepared using Alcalase showed the increase in chelating activity with increasing DH, but those treated with Flavourzyme showed no difference in activity at all DH tested (Thainsilakul *et al*., 2007a). With the same enzymes used, chelating activity of protein hydrolysate prepared from the muscle of yellow stripe trevally using Flavourzyme and Alcalase increased with increasing DH (Klompong *et al*., 2007). Higher ferrous chelating activity was reported for hydrolysate of silver carp using Alcalase and Flavourzyme when DH increased (Dong *et al*., 2008). Apart from Fe, other transition metals, such as Cu and Co can affect the rate of lipid oxidation and decomposition of hydroperoxide. Theodore *et al*. (2008) reported that Cu^{2+} chelating activity of catfish protein hydrolysate incresed with increasing DH.

Some proteins and peptides can chelate metal ions like Fe^{2+} due to the presence of carboxyl and amino groups in the side chains of acidic and basic amino acids (Saiga *et al*., 2003; Samaranayaka and Li-Chan, 2008). Alcalase is endopeptidase capable of hydrolyzing proteins with broad specicity for peptide bonds and prefers for the uncharged residue, whereas Flavourzyme is the endo-and exopeptidase enzyme mixture, which can produce both amino acids and peptides (Klompong *et al*., 2008). The antioxidative activity of hydrolysates treated with different enzymes might result in the differences in the exposed side chains of peptides as governed by the specificity of proteases towards peptide bonds in the proteins (Bayram *et al*., 2008). DH also greatly influenced the peptide chain length. The higher DH, the more cleavage of peptide chains took place. Peptides with various sizes and compositions had various capacities of scavenging or quenching free radicals (Thainsilakul *et al*., 2007a; Klompong *et al*., 2007). PCP, Alcalase and Flavourzyme more likely cleaved the peptide bonds in brownstripe red snapper muscle at different positions, resulting in the different products with varying

antioxidative activities. With 40% DH, all hydrolysates functioned as the primary antioxidant more effectively, whereas the secondary antioxidative activity, the metal chelating, was lowered. To enhance the antioxidative activity of peptides, especially as the primary antioxidant, the hydrolysate with DH of 40% was prepared for the first step of hydrolysis.

5.4.2 Antioxidative and ACE inhibitory activities of brownstripe red snapper protein hydrolysate prepared with two-step hydrolysis using different proteases

5.4.2.1 DPPH radical scavenging activity

DPPH radical scavenging activity of protein hydrolysates with various hydrolysis times in the second step of hydrolysis using another protease is shown in Figure 21. For HAF, HAP, HFA and HFP, the activities increased with increasing time up to 2 h ($p < 0.05$). Thereafter, the gradual decrease was observed when hydrolysis times were 3 and 5 h ($p < 0.05$). No changes in DPPH radical scavenging activity was observed from HPF, while HPA showed the maximal activity at 3 h of hydrolysis ($p < 0.05$). The results suggested that scavenging activity against DPPH radical was enhanced by additional hydrolysis using another enzyme with an appropriate time. Generally, hydrolysates prepared by further hydrolyzing the hydrolysate obtained from the first step of hydrolysis with another protease for 2 h had a greater scavenging ability against DPPH radical.

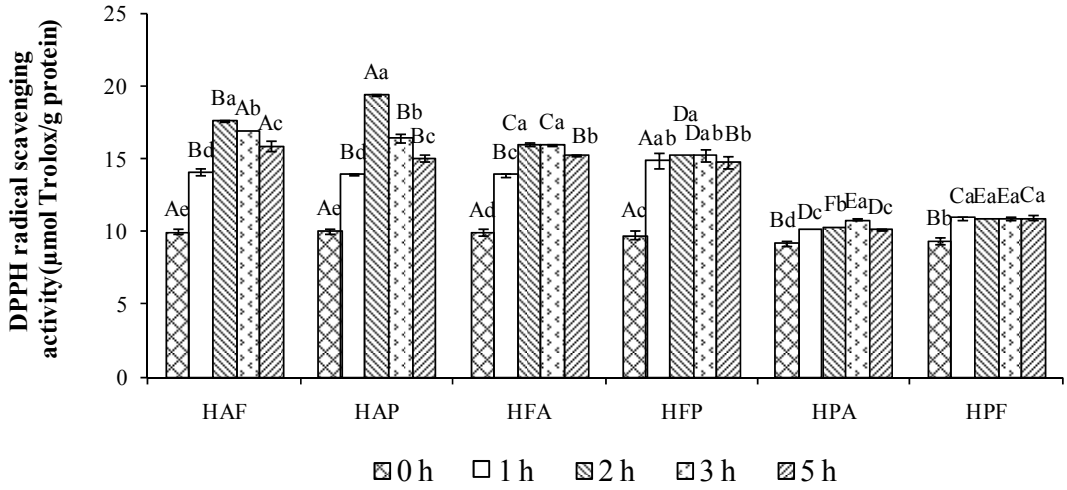

Figure 21. DPPH radical scavenging activities of protein hydrolysate from brownstripe red snapper muscle prepared by two-step hydrolysis process with different hydrolysis times in the second step of hydrolysis. HAF and HAP: HA hydrolyzed with Flavourzyme and PCP, respectively. HFA and HFP: HF hydrolyzed with Alcalase and PCP, respectively. HPA and HPF: HP hydrolyzed with Alcalase and Flavourzyme, respectively. Bars represent standard deviation (n=3). Different capital letters within the same hydrolysis time and different letters within the same treatment indicate significant differences ($p < 0.05$).

5.4.2.2 ABTS radical scavenging activity

ABTS radical scavenging activity of protein hydrolysates prepared by two-step hydrolysis is shown in Figure 22. Only HAF and HAP showed the increases in ABTS radical scavenging activity, compared with their parent counterparts, HA. HAP with the hydrolysis time of 2 h for the second step had the highest activity ($p < 0.05$). This suggested the enhancement of ABTS radical scavenging activity when PCP was used for further hydrolysis of HA. However, the further hydrolysis of HF with another protease had no impact on the increases in activity ($p > 0.05$). For HP, the decrease in activities was obtained when the second step of hydrolysis was performed ($p < 0.05$). The results suggested that the second step of hydrolysis could slightly increase ABTS radical scavenging activity, depending on the first hydrolysate as well as the types of protease used for the second step. It was noted that the ability

of scavenging ABTS and DPPH radical by hydrolysates were different. This might be due to the differences in ability of scavenging the different radicals, ABTS and DPPH, by the same peptide.

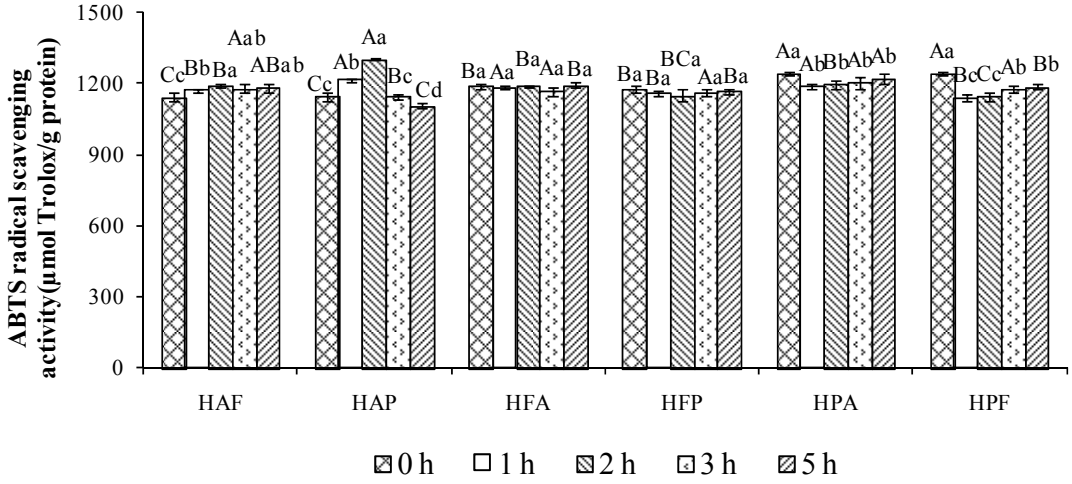

Figure 22. ABTS radical scavenging activities of protein hydrolysate from brownstripe red snapper muscle prepared by two-step hydrolysis process with different hydrolysis times in the second step of hydrolysis. HAF and HAP: HA hydrolyzed with Flavourzyme and PCP, respectively. HFA and HFP: HF hydrolyzed with Alcalase and PCP, respectively. HPA and HPF: HP hydrolyzed with Alcalase and Flavourzyme, respectively. Bars represent standard deviation (n=3). Different capital letters within the same hydrolysis time and different letters within the same treatment indicate significant differences ($p < 0.05$).

5.4.2.3 Ferric reducing antioxidant power (FRAP)

Figure 23 shows FRAP of protein hydrolysates prepared using two-step hydrolysis. The increases in FRAP were observed for all hydrolysis when the second step of hydrolysis was applied. For HF and HP, when Alcalase was used for the second step of hydrolysis, the continuous increase in FRAP were observed ($p < 0.05$). For other hydrolysates, the hydrolysis time was the factor affecting the FRAP of resulting hydrolysate, depending on the first hydrolysate and the types of protease

used for the second step of hydrolysis. The increase in FRAP with increasing hydrolysis time was coincidental with the increase in DPPH radical scavenging activity (Figure 21). For HAF, HAP and HPF, the activities were increased with increasing hydrolysis time up to 2 h ($p < 0.05$). For HFP, the hydrolysis time more than 1 h had the negative effect on FRAP ($p < 0.05$). Generally, further hydrolysis with another protease led to the increase in FRAP, but the activities of resulting hydrolysate were governed by hydrolysis time.

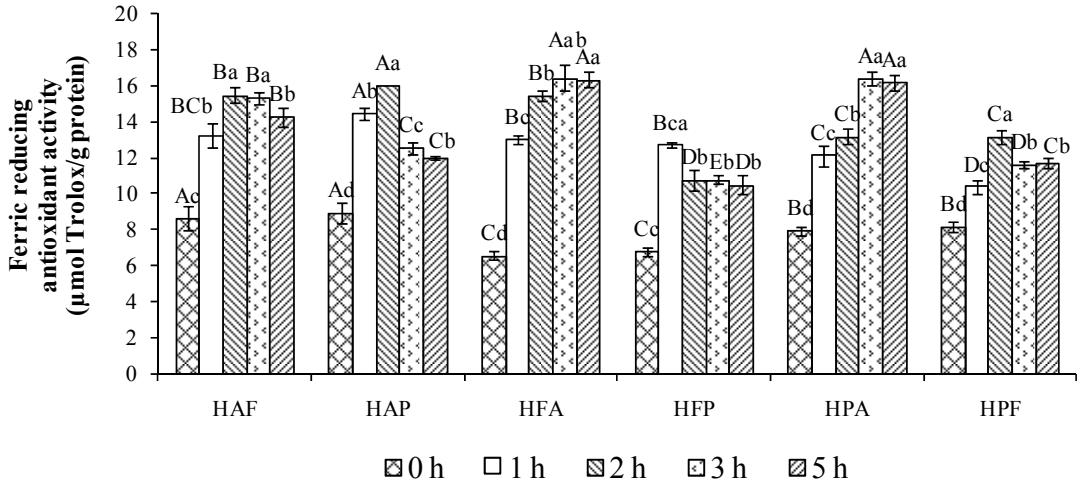

Figure 23. Ferric reducing antioxidant power (FRAP) of protein hydrolysate from brownstripe red snapper muscle prepared by two-step hydrolysis process with different hydrolysis times in the second step of hydrolysis. HAF and HAP: HA hydrolyzed with Flavourzyme and PCP, respectively. HFA and HFP: HF hydrolyzed with Alcalase and PCP, respectively. HPA and HPF: HP hydrolyzed with Alcalase and Flavourzyme, respectively. Bars represent standard deviation (n=3). Different capital letters within the same hydrolysis time and different letters within the same treatment indicate significant differences ($p < 0.05$).

5.4.2.4 Ferrous chelating activity

Ferrous chelating activities of hydrolysates prepared by two-step hydrolysis are shown in Figure 24. HFA and HPA showed the marked decrease in

chelating activities when the second hydrolysis was conducted ($p < 0.05$). Alcalase used in the second step might generate peptides with the lower ability in Fe^{2+} chelating. The abilities of HAF, HAP, HFP and HPF in chelating Fe^{2+} ion were more pronounced, compared with their parent hydrolysate counterparts. The increases were observed when a certain time of the second hydrolysis was used. Among all protein hydrolysates, HFP showed the obviously high ferrous chelating activity, especially when the second step hydrolysis time of 1 h was used ($p < 0.05$). The differences in antioxidative activities among protein hydrolysates prepared using two-step hydrolysis might be governed by the different resulting peptides in hydrolysates.

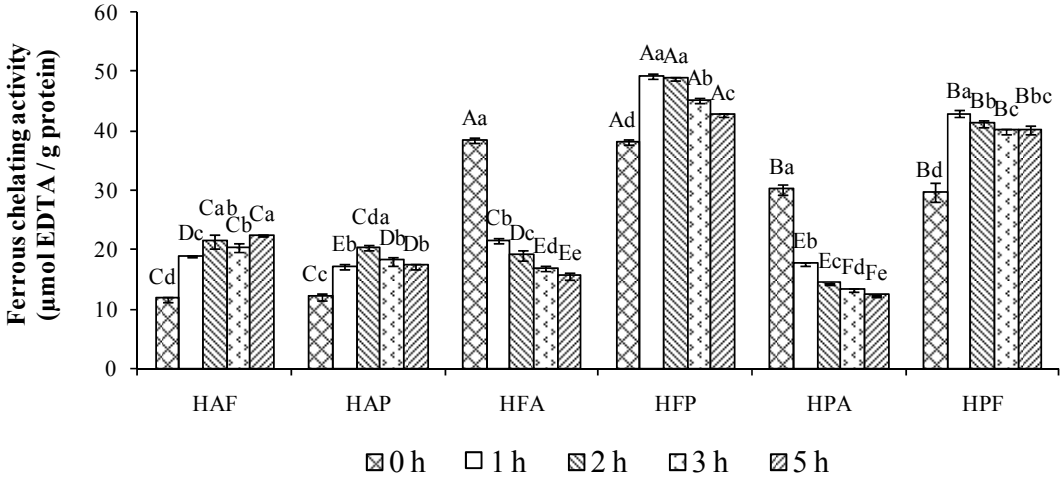

Figure 24. Ferrous chelating activity of protein hydrolysate from brownstripe red snapper muscle prepared by two-step hydrolysis process with different hydrolysis times in the second step of hydrolysis. HAF and HAP: HA hydrolyzed with Flavourzyme and PCP, respectively. HFA and HFP: HF hydrolyzed with Alcalase and PCP, respectively. HPA and HPF: HP hydrolyzed with Alcalase and Flavourzyme, respectively. Bars represent standard deviation (n=3). Different capital letters within the same hydrolysis time and different letters within the same treatment indicate significant differences ($p < 0.05$).

5.4.3 ACE inhibitory activity

The inhibitory effect of all hydrolysates prepared using two-step hydrolysis against angiotensin I-convering enzyme (ACE) was determined as shown in Figure 25. For the first hydrolysate prepared using the single different proteases (0 h), HF showed the highest ACE inhibition (11.44 %) ($p < 0.05$). Flavourzyme might produce the peptides with ACE inhibitory activity. Raghavan and Kristinsson (2009) used Cryotin and Flavourzyme for hydrolysis of tilapia protein and found the higher ACE inhibition by hydrolysate prepared using Flavourzyme. When the second step of hydrolysis using another protease was conducted, HFA and HFP showed the higher ACE inhibition when hydrolysis time of 2 and 3 h was used, respectively. HAP also showed high ACE inhibitory activity. Wu *et al*. (2008) reported that shark meat treated with enzyme showed the higher ACE inhibition than that of untreated one. ACE inhibition by tilapia protein hydrolysate was reported, especially with increasing DH (Raghavan and Kristinsson, 2009). Furthermore, smaller peptides are the better ACE inhibitors than the larger counterpart (Raghavan and Kristinsson, 2009). The hydrolysis might release ACE inhibitory peptides in hydrolysate (Wu *et al*., 2008). Hydrolysates from different sources have been reported to possess ACE inhibitory activity, e.g. hydrolysate from freshwater calm muscle (Tsai *et al*., 2006), shark meat (Wu *et al*., 2008), Atlantic salmon, Coho salmon, Alaska Pollack and southern blue whiting muscle (Nakajima *et al*., 2009).

From the results, the use of Alcalase for the first hydrolysis and the use of PCP in the second hydrolysis for 2 h (HAP) yielded the resulting hydrolysate with the higher DPPH and ABTS radical scavenging activities and FRAP, compared to other hydrolysates ($p < 0.05$). However HF hydrolyzed with PCP in the second hydrolysis step for 1 h (HFP) showed much higher chelating activity, compared with others ($p < 0.05$). Therefore, HFP and HAP prepared with 1 and 2 h for the second step of hydrolysis, respectively, were selected for further study. DH of selected protein hydrolysates, HAP and HFP, were 62.7 and 61.8 %, respectively.

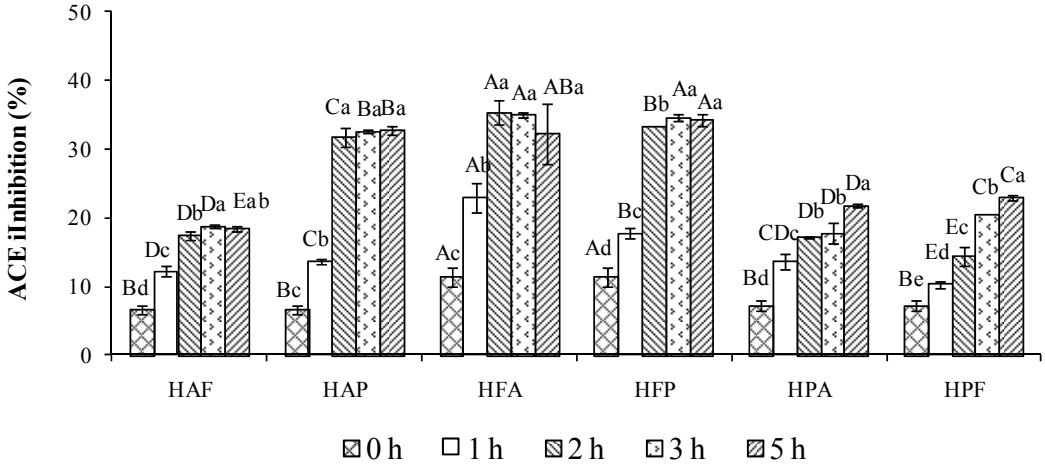

Figure 25. ACE inhibitory activity of protein hydrolysate from brownstripe red snapper muscle prepared by two-step hydrolysis process with different hydrolysis times in the second step of hydrolysis. HAF and HAP: HA hydrolyzed with Flavourzyme and PCP, respectively. HFA and HFP: HF hydrolyzed with Alcalase and PCP, respectively. HPA and HPF: HP hydrolyzed with Alcalase and Flavourzyme, respectively. Protein concentration used for ACE inhibitory assay was 5 mg/mL. Bars represent standard deviation (n=3). Different capital letters within the same hydrolysis time and different letters within the same treatment indicate significant differences ($p < 0.05$).

5.4.4 Antioxidative activities of brownstripe red snapper protein hydrolysate in different oxidation model systems

5.4.4.1 *β*-carotene linoleic acid emulsion model system

Selected protein hydrolysates, HAP and HFP, were studied on their antioxidative activity in *β*-carotene linoleic acid oxidation model system as shown in Figure 26. The decrease in A_{470} indicates the oxidation of *β*-carotene in the system caused by the oxidation process. When the oxidation of linoleic acid occurs, free radicals formed are able to attack the highly unsaturated *β*-carotene molecules. As a result, *β*-carotene is oxidized, leading to the losses in chromophore and characteristic

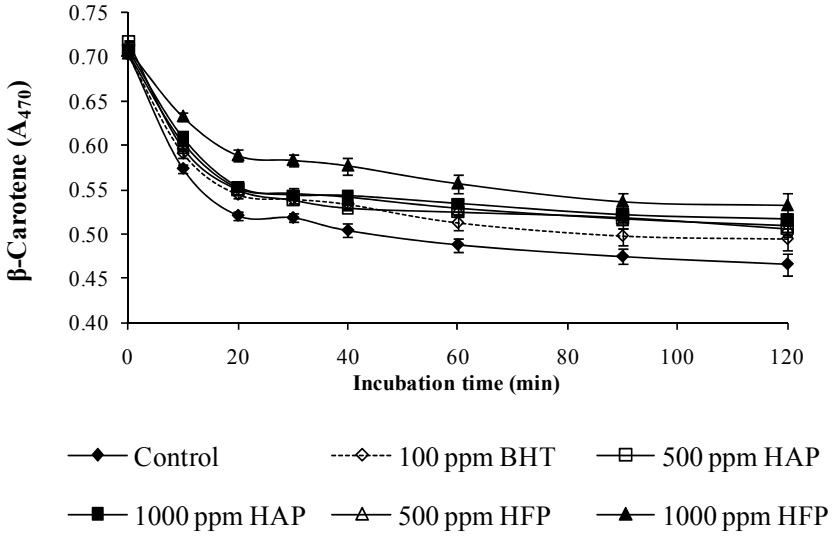

Figure 26. Changes in A_{470} of *β*-carotene linoleic acid system in the absence or the presence of protein hydrolysate from brownstripe red snapper muscle at different levels. HAP: HA hydrolyzed with PCP. HFP: HF hydrolyzed with PCP. BHT is butylated hydroxytoluene. Bars represent the standard deviation from triplicate determinations.

orange color of *β*-carotene (Binson *et al*., 2008). The presence of antioxidant in linoleic acid emulsion system hinders *β*-carotene bleaching, due to the chain-breaking inhibition of lipid peroxidation by neutralizing the linoleic free radicals formed. Systems containing HAP and HFP at levels of 500 and 1,000 ppm retarded the decrease in A_{470} up to 120 min ($p < 0.05$). However, no difference in A_{470} was observed between the system containing 100 ppm BHT and those with 500 and 1,000 ppm HAP and 500 ppm HFP within the first 40 min ($p > 0.05$). This indicated that the preventive effect of hydrolysates (500 and 1,000 ppm) was equivalent to that of 100 ppm BHT. Thereafter, the lower *β*-carotene bleaching in the systems containing these hydrolysates were observed, compared with the system added with 100 ppm BHT up to 120 min. The result indicated that in emulsion system, HAP and HFP could act as the better chain breaker than BHT, especially with increasing reaction time. Among the hydrolysates, 1,000 ppm HFP showed the highest inhibitory activity against *β*-carotene bleaching throughout the reaction time of 120 min ($p < 0.05$). The ability of

hydrolysates to prevent the bleaching of *β*-carotene was more likely governed by their amphiphilic properties of amino acids compositions. Localization/orientation at the interface of hydrophobic part of peptides to oil phase and of hydrophilic portion to aqueous phase at the interface is the major principle for emulsion stabilization (Binson *et al*., 2008). The result suggested that protein hydrolysates tested contained peptides with both hydrophilic and hydrophobic portions. As a result, the antioxidative activity could be maximized at the interface of emulsion.

5.4.4.2 Lecithin liposome model system

HAP and HFP at level of 500 and 1,000 ppm affected the oxidation of the lecithin liposome system differently as indicated by different conjugated dienes (CD) (Figure 27A) and TBARS values (Figure 27B). All systems containing hydrolysate samples, except for that added with 100 ppm BHT, showed the similar pattern of CD formation, in which the increase was found within the first 12-18 h of reaction. Thereafter, the decrease in CD was observed. However, the rate of changes varied with the hydrolysates added and concentration used. In the early incubation time (0-6 h), the delay in CD formation was observed. System containing 1000 ppm HFP showed the longer delay time, compared with systems containing other hydrolysates, indicating the higher resistance to oxidation of the system (Thainsilakul *et al*., 2007). After 6 h, the remarkable increases in CD were observed, especially within the first 18 h ($p < 0.05$). Within the first 6-12 h of incubation, the increasing rate of CD in the system varied with the hydrolysates and the concentrations used. At the same concentration used, the liposome system added with HFP showed the lower CD formation as evidenced by lower A_{234} and the systems containing higher concentration of hydrolysates showed the lower CD formation ($p < 0.05$). When comparing with the control, liposome system added with 500 and 1000 ppm HFP and 1000 ppm HAP had the lower CD than the control ($p <0.05$), indicating the ability to retard the formation of CD of those hydrolysates (Klompong *et al*., 2008). However, after 18 h, CD of all sample added with hydrolysates was higher than that of the control and gradually decreased up to 36 h. Subsequently, CD was quite stable throughout 60 h of incubation. The decrease in CD was probably due to the

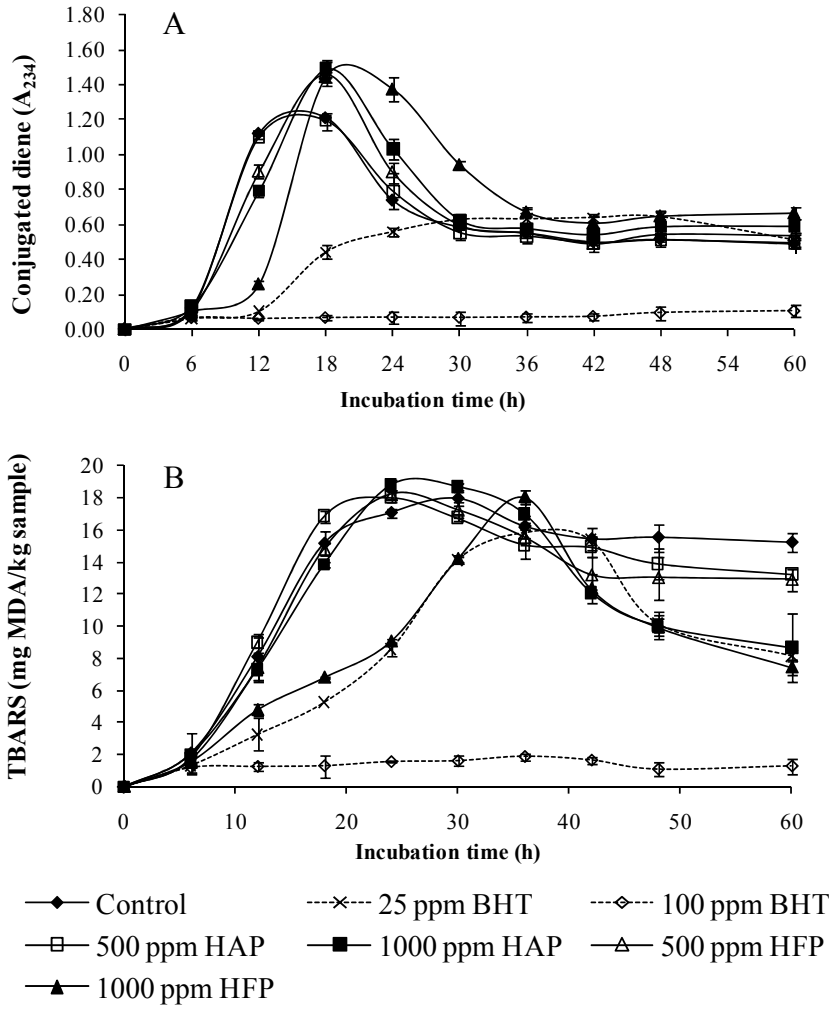

Figure 27. Changes in conjugated diene (A) and thiobarbituric acid reactive substances (TBARS) value (B) in the lecithin liposome system in the absence or the presence of protein hydrolysate from brownstripe red snapper muscle at different levels. HAP: HA hydrolyzed with PCP. HFP: HF hydrolyzed with PCP. BHT is butylated hydroxytoluene. Bars represent the standard deviation (n=3).

transformation of CD into hydroperoxide by oxidation process. Frankel *et al*. (1997) reported that the lower CD content could be caused by the higher formation of hydroperoxides in propagation stage and these compounds are prone to decomposition to secondary oxidation products, which can be determined by TBARS value. The result indicated that hydrolysates might retard the decomposition of primary products

into the secondary ones as evidenced by the higher CD in samples added with hydrolysate than that of the control. However, no changes in CD of liposome system added with 100 ppm BHT was observed throughout 60 h of incubation ($p > 0.05$). For the system added with 25 ppm BHT, the increase in CD was found after 12 h of incubation and reached the plateau at h 36.

The formation of secondary lipid oxidation products was evaluated by TBARS. The sharp increases in TBARS were observed within the first 24 h of incubation when 500 and 1,000 ppm HAP and 500 ppm HFP were added to liposome system. However, the system added with 1,000 ppm HFP showed the lower TBARS than those added with other hydrolysates. it was noted that system added with 1,000 ppm HFP had the similar TBARS, compared with that containing 25 ppm BHT. The early formation of TBARS indicated the decomposition of the primary products to the secondary products. After the particular incubation time, the gradual decreases in TBARS were observed, possibly due to the loss of volatile compounds produced in those systems. HFP, especially at 1,000 ppm, showed the noticeable antioxidative activity in liposome system throughout the 60 h of incubation time as evidenced by the lower TBARS formation ($p < 0.05$). The result suggested the greater ability of 1,000 ppm HFP in retardation of the formation of secondary oxidation products. Liposome system added with 100 ppm BHT showed no changes in TBARS throughout 60 h of incubation ($p > 0.05$).

Liposomes are appropriate lipid models to evaluate antioxidative activity in lipid food or lipoprotein particles containing phospholipids (Frankel *et al*., 1997). Polar portions of peptides in hydrolysate might interact with the polar environment of lecithin liposomes, resulting in the better protection against lipid oxidation at the interface. It could be concluded that protein hydrolysates, especially 1,000 ppm HFP, could retard the early stages of lipid oxidation (formation of CD) as well as the propagation of oxidation process (decomposition of CD to TBARS) via their radical scavenging activity, reducing power and metal chelating activity.

5.4.4.3 Comminuted fish model system

When HAP and HFP at a level of 1,000 ppm were added in the mince from two fish species including brownstripe red snapper and round scad, TBARS were monitored during the storage at 4°C for 10 days in comparison with the control and that containing 200 ppm BHT as shown in Figure 28A and B, respectively. TBARS values of both mince samples, except those added with 200 ppm BHT, increased up to 6 days of storage, followed by the decrease during 8-10 days of storage. When comparing between both fish mince systems, brownstripe red snapper mince had the lower TBARS than round scad. This was due to the fact that round scad is dark-meat fish containing higher amount of fat and protein-bound iron such as myoglobin (Artharn *et al*., 2007), which might act as pro-oxidants. Free radicals could abstract hydrogen from fatty acid double bond and new radicals were produced and further reacted with oxygen to produce fatty acid hydroperoxides. These unstable compounds could be decomposed readily to shorter chain hydrocarbons which can be determined as TBARS (Benjakul *et al*., 2005a). Among all samples, the one containing 200 ppm BHT had the much lower TBARS values throughout the storage time ($p < 0.05$). At day 10 of storage, the control had the highest TBARS, whereas those added with HAP and HFP at a level of 1000 ppm possessed the lower TBARS, suggesting the antioxidative activity of both HAP and HFP in the mince to some degrees. Comparing between this two hydrolysates, HAP was able to retard lipid oxidation more effectively than did HAP ($p < 0.05$). Owing to the higher chelating activity of HFP, it might retard the lipid oxidation by scavenging of Fe^{2+} ion, the most reactive pro-oxidant metal ion (Yomauchi *et al*., 1988) along with its radical scavenging ability.

Benjakul *et al*. (2005a) used comminuted Pacific saury (*Cololabis saira*) system to investigate the prevention of lipid oxidation by caramelisation products (CPs) and reported that CPs could retard the formation of TBARS in mince system tested. Mungoong, a extract paste from cephalothorax of white shrimp (*Litopenaeus vannamei*) with the aid of Flavourzyme, showed the inhibitory activity against lipid oxidation in round scad mince system in a dose-dependent manner (Binson *et al*., 2008). In food-related systems, antioxidant activity is mainly due to the

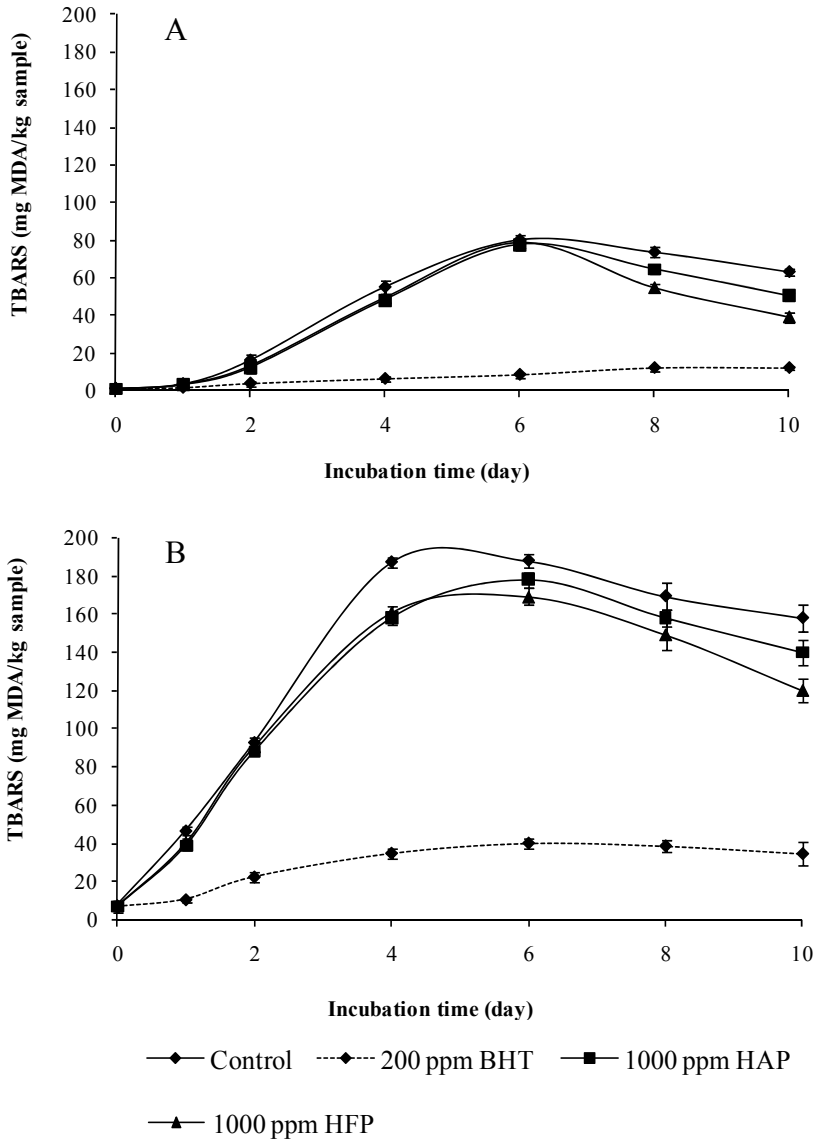

Figure 28. Changes in TBARS value of comminuted brownstripe red snapper mince model system (A) and round scad mince model system (B) in the absence or the presence of protein hydrolysate from brownstripe red snapper muscle. HAP: HA hydrolyzed with PCP. HFP: HF hydrolyzed with PCP. BHT is butylated hydroxytoluene. Bars represent the standard deviation (n=3).

scavenging of free radical and the chain-breaking inhibition of lipid oxidation (Santoso *et al*., 2004; Podsedek, 2007). Therefore, the hydrolysate from brownstripe red snapper could retard lipid oxidation *in vitro* assay systems and oxidation model systems. The results suggested that hydrolysate from brownstripe red snapper protein could function as a natural antioxidant in food or functional food.

5.4.5 Changes in antioxidative activity in gastrointestinal tract model system (GIMs)

GIMs was used to simulate the ingestion system of human body and the remaining antioxidative activities of HAP and HFP were monitored. When protein hydrolysate was oral administrated, their bioactive peptides should be resistant to hydrolysis by digestive proteases in order to be absorbed and reach the target organ to function as antioxidant (Burkitt, 2001). Both HAP and HFP showed the similar activities in GIMs. Both hydrolysates showed the increase in DPPH radical scavenging activity within the first hour of digestion. Thereafter, during intestinal simulated system, DPPH radical scavenging activities were similar to that of original hydrolysate and remained constant up to 240 min. On the other hand, the increase in FRAP was found for both HAP and HFP during the first hour of digestion ($p < 0.05$). The further increases were also found in the intestinal simulated system up to 120 and 100 min for HAP and HFP, repectively. Thereafter, no changes in FRAP were found up to 240 min for HAP, whereas the slight decrease up to 240 min was observed for HFP ($p > 0.05$). Nevertheless, no changes in both ABTS radical scavenging and ferrous chelating activities were found within the first hour (stomach simulated system) ($p > 0.05$). With further hydrolysis in intestinal simulated system, the sharp increases in both ABTS radical scavenging and ferrous chelating activities were obtained but no changes in activity were found with increasing digestion time up to 240 min ($p > 0.05$).

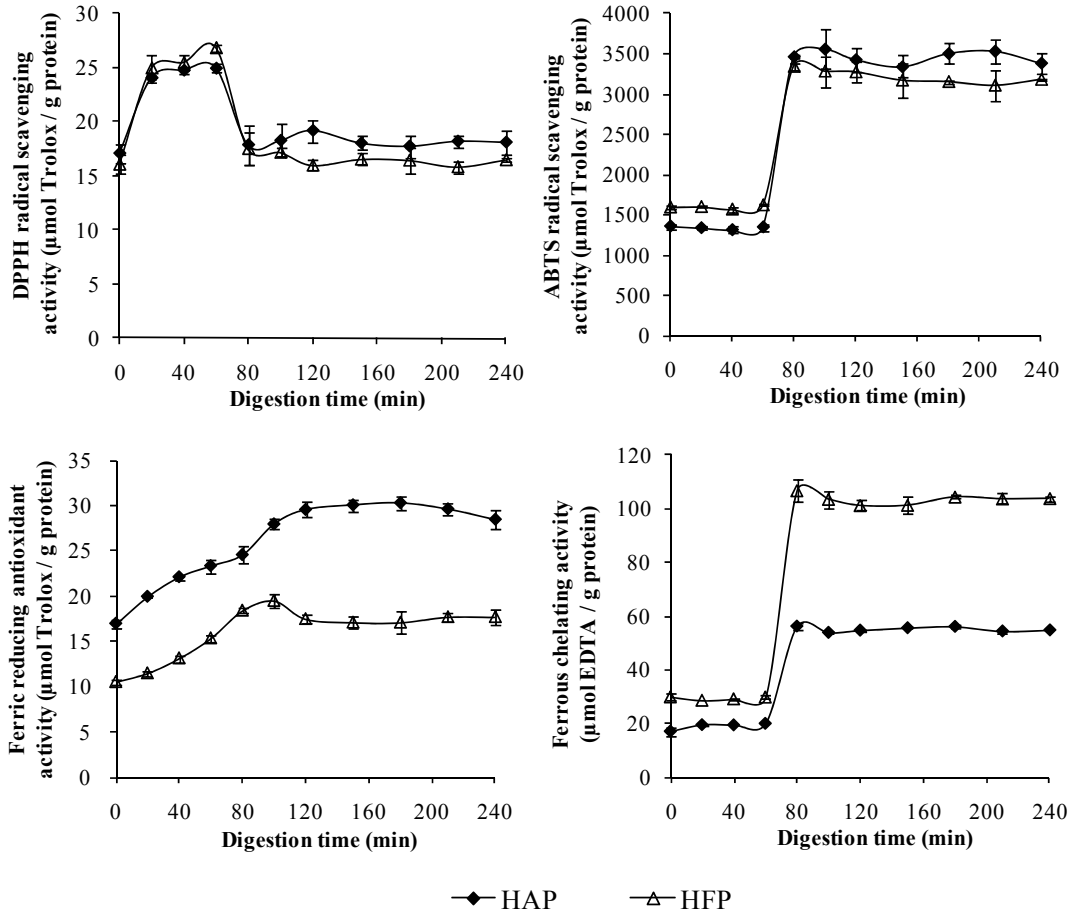

Figure 29. Antioxidative activities of protein hydrolysate from brownstripe red snapper muscle prepared by two-step hydrolysis process in gastrointestinal tract model system. HAP: HA hydrolyzed with PCP. HFP: HF hydrolyzed with PCP. Bars represent standard deviation (n=3).

Pepsin might hydrolyze HAP and HFP to some degrees, yielding the new peptides with DPPH radical scavenging activity and reducing power. It was noted that HAP showed the higher FRAP than HFP during the first hour of digestion. Further digestion with pancreatin (intestinal simulated system) caused the dramatic increase in ABTS radical scavenging activity, FRAP and ferrous chelating activity, but with different rates ($p < 0.05$). During pancreatin digestion, HAP showed the higher DPPH, ABTS radical scavenging activities and FRAP, but lower ferrous chelating activity, compared with HFP. 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, GIMs may actually lead to generation of more potent bioactive peptides (Megias *et al*., 2009). The antioxidative activities of protein hydrolysates after incubation in GIMs was dependent on peptides in hydrolysate, in term of size, amino acid composition and sequence, which could be targeted by digestive proteases (Megias *et al*., 2009). The results showed that the antioxidative activity of both HAP and HFP could be maintained after treatment with these gastrointestinal enzymes in GIMs ($p < 0.05$). Thus, both HAP and HFP were more likely resistant to digestion in the real gastrointestinal tract of human body.

5.5 Conclusion

Protein hydrolysates prepared from the muscle of brownstripe red snapper using protease from its pyloric caeca in combination with commercial enzymes could provide the peptides with antioxidative and ACE inhibitory activities. Protein hydrolysates prepared using Alcalase and Flavourzyme at the first step, followed by further hydrolysis using protease from its pyloric caeca showed the highest antioxidative activities, both *in vitro* and oxidation model systems. These hydrolysates can function as natural antioxidant in comminuted fish mince. Antioxidative activities of protein hydrolysates remained constant or exhibited the stronger antioxidative activities after digestion in gastrointestinal tract model system. Therefore, hydrolysate from brownstripe red snapper muscle by two-step hydrolysis could serve as the natural antioxidant for food preservation or as the functional foods.

CHAPTER 6

ISOLATION AND IDENTIFICATION OF ANTIOXIDATIVE PEPTIDES FROM BROWNSTRIPE RED SNAPPER PROTEIN HYDROLYSATE

6.1 Abstract

Hydrolysates from the muscle of brownstripe red snapper (*Lutjanus vitta*) prepared using 2-step hydrolysis by Alcalase/pyloric caeca protease (PCP) (HAP) and Flavourzyme/PCP (HFP) were characterized and identified for their antioxidative peptides. HAP showed the higher ABTS and DPPH radical scavenging activities and ferric reducing antioxidant power than did HFP, whereas the higher ferrous chelating activity was observed for HFP ($p < 0.05$). Antioxidative activity of HAP and HFP fractions separated using a series of chromatographies, including Sephadex G-25 column and Phenogel^{TM} column was monitored. The fractions exhibiting the highest antioxidative activity were selected for mass analysis using LC-MS, the MS/MS data of fragmented peptides were analyzed by DeCyderMS and the amino acid sequence of each peptide was identified by PEAK online Software. Peptides showing ABTS radical scavenging activities in HAP were identified as Asn-Arg-Lys-Arg, Asp-Ala-Gly-Leu-Phe-Lys, Met-Glu-Glu-Asp-Leu-Glu-Glu-Arg and Met-Ser-Leu-Trp-Gln-Ser-Leu-Met-Asn-Asp-Lys. Ferrous chelating peptides derived from HFP were Cys-Gly-Asp-Ser-Val-Lys, Met-Cys-Cys-Cys-Arg, His-Arg-Arg-Arg, Asn-Phe-Cys-Ser-Arg, Trp-Trp-Arg-Lys and Phe-Cys-Gly-Val-Ala-Thr-Lys.

6.2 Introduction

Fish protein hydrolysates prepared from enzymatic hydrolysis, are the best among protein hydrolysates, in term of nutritional properties, balanced amino acid composition and high digestibility (Kristinsson and Rasco, 2000b; Picot *et al*., 2006). Protein hydrolysates from various fish have been reported to possess antioxidant activities such as protein hydrolysate from mackerel (*Scomber austriasicus*) (Wu *et al*., 2003), red salmon (*Oncorhynchus nerka*) (Sathivel *et al*., 2005), round scad (*Decapterus maruadsi*) (Thiansilakul *et al*., 2007a), yellow stripe trevally (*Selaroides leptolepis*) (Klompong *et al*., 2007) and Pacific hake (*Merluccius productus*) (Samaranayaka and Li-Chan 2008). Various protein hydrolysates prepared from different fish species or different proteases used contained varying active peptides. Arg-Pro-Asp-Phe-Asp-Leu-Glu-Pro-Pro-Tyr was identified as antioxidative peptide in yellowfin sole frame protein hydrolysate (Jun *et al*., 2004). Alaska pollack frame protein was hydrolyzed and the antioxidative peptide was identified as Leu-Pro-His-Ser-Gly-Tyr with a molecular weight of 672 Da (Je *et al*., 2005). Kim *et al*. (2007) isolated an antioxidative peptide from hoki frame protein hydrolysate and the peptide was identified as Glu-Ser-Thr-Val-Pro-Glu-Arg-Thr-His-Pro-Ala-Cys-Pro-Asp-Phe-Asn with a molecular weight of 1801 Da. Val-Lys-Ala-Gly-Phe-Ala-Trp-Thr-Ala-Asn-Gln-Gln-Leu-Ser (1519 Da) was identified as antioxidative peptide in tuna backbone protein hydrolysate (Je *et al*., 2007). Additionally, by-products from fishery processing were also isolated and identified for their antioxidative peptide. Antioxidative peptides isolated from hydrolyzed tuna cooking juice were Pro-Val-Ser-His-Asp-His-Ala-Pro-Glu-Tyr (1305 Da), Pro-Ser-Asp-His-Asp-His-Glu (938 Da), and Val-His-Asp-Tyr (584 Da) (Hsu *et al*., 2009). The antioxidative peptide from heads and viscera proteins of sardinelle (*Sardinella aurita*) was Leu-His-Tyr (Bougatef *et al*., 2010b).

In Thailand, brownstripe red snapper (*Lutjanus vitta*) has been served as raw materials for surimi production (Khantaphant and Benjakul, 2008) (Chapter 2). Apart from being processed into surimi, this species can be produced as raw material for production of protein hydrolysates with bioactivities, especially antioxidant

activity. The use of two-step hydrolysis with different proteases has been recently reported to enhance antioxidative activity of hydrolysate from brownstripe red snapper (Chapter 5). However, the identification of antioxidative peptides from brownstripe red snapper hydrolysate has not been conducted. This work aimed to isolate and identify antioxidative peptides in hydrolysate from brownstripe red snapper muscle.

6.3 Materials and Methods

6.3.1 Chemicals

Alcalase 2.4 L (E.C 3.4.21.62) (2.4 AU/g) and Flavourzyme 500 L (E.C 3.4.21.77) (500 LAPU/g) were provided by Novozyme (Bagsvaerd, Denmark). 1,1-diphenyl-2-picrylhydrazyl (DPPH), 2,4,6-tripyridyl-triazine (TPTZ), 2,2′-azinobis(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS) and 3-(2-pyridyl)-5, 6-diphenyl-1, 2, 4-triazine-4′, 4″-disulfonic acid (ferrozine) were purchased from Sigma (St. Louis, MO, USA). Tris (hydroxymethyl) aminomethane (Tris-HCl) was procured from Merck (Darmstadt, Germany) and sodium sulfite was obtained from Riedel-deHaën (Seelze, Germany). All chemicals were of analytical grade.

6.3.2 Preparation of pretreated fish mince

Brownstripe red snapper, off-loaded approximately 24-36 h after capture, were purchased from a dock in Songkhla province, Thailand. Fish were transported in ice with the fish/ice ratio of 1:2 (w/w) to the Department of Food Technology, Prince of Songkla University within 1 h. Upon arrival, whole fish were washed and only flesh was separated manually. Flesh was minced to uniformity using Moulinex AY46 blender (Group SEB, Lyon, France) before homogenizing with nine volumes of cold 8 mM $CaCl_2$ solution containing 5 mM citric acid using an IKA Labortechnik homogenizer (Selangor, Malaysia) at a speed of 11,000 rpm for 2 min. After continuous stirring for 60 min at 4°C, the sample was centrifuged at 4,000 x *g*

for 15 min at 4°C using a Beckman Coulter centrifuge Model Avant J-E (Beckman Coulter, Inc., Fullerton, CA, USA). Thereafter, the pellet was washed by homogenizing with five volumes of cold distilled water using a homogenizer at a speed of 11,000 rpm for 2 min, followed by stirring at 4°C for 15 min prior to centrifuging at 9,600 x *g* for 10 min at 4°C. The washing process was repeated twice. Pretreated mince obtained was kept in polyethylene bags and placed in ice until use, but not longer than 2 h.

6.3.3 Preparation of proteases from pyloric caeca

Pyloric caeca from brownstripe red snapper was collected and powdered in liquid nitrogen. Thereafter, the pyloric caeca extract was prepared according to the method of Khantaphant and Benjakul (2010) (Chapter 3). Pyloric caeca powder was suspended in ten volumes of 50 mM Tris-HCl buffer, pH 8.0, containing 10 mM $CaCl_2$. The mixture was homogenized at 11,000 rpm for 2 min. The homogenate was continuously stirred for 30 min at 4°C and centrifuged at 8,000 x *g* for 30 min at 4°C. The supernatant was filtered through a Whatman filter paper No. 1 (Whatman International Ltd., Maidstone, England). The filtrate obtained was further subjected to 40-60% saturation ammonium sulfate precipitation. After stirring at 4°C for 30 min, the mixture was centrifuged at 8,000 x *g* for 30 min at 4°C and the pellet obtained was dissolved in 50 mM Tris-HCl buffer, pH 8.0 followed by dialysis 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 'pyloric caeca protease; PCP'. PCP was determined for proteolytic activity using casein as a substrate under optimal condition (60°C and pH 8.5).

6.3.4 Preparation of brownstripe red snapper protein hydrolysates

Pretreated brownstripe red snapper mince was mixed with 50 mM Tris-HCl buffer, pH 7.0 and 8.0 for hydrolysis using Flavourzyme and Alcalase, respectively, to obtain a final protein concentration of 2% (w/v). The mixtures were

homogenized at a speed of 11,000 rpm for 1 min and the homogenates were pre-incubated at 50°C for 10 min. The hydrolysis reaction was initiated by adding Alcalase or Flavourzyme at the amount required to gain DH of 40% following the method of Benjakul and Morrissey (1997). After 2 h of hydrolysis, the reactions were inactivated by heating the mixture in boiling water for 10 min. The mixture was then centrifuged at 2,000 x *g* at 4°C for 10 min and the supernatant was collected and referred to as protein hydrolysate prepared using Alcalase (HA) and Flavourzyme (HF). The further hydrolysis by PCP from brownstripe red snapper was conducted at pH 8.5 and 60°C for 1 and 2 h for HF and HA, respectively. The hydrolysis was finally terminated by placing the mixture in boiling water for 10 min followed by centrifugation at 2,000 x *g* at 4°C for 10 min. The supernatant was collected and lyophilized. The lyophilized brownstripe red snapper protein hydrolysates prepared using Alcalase/PCP and Flavourzyme/PCP were referred to as 'HAP' and 'HFP', respectively. The obtained hydrolysates were determined for antioxidative activities and further subjected to isolation and identification for antioxidative peptides.

6.3.5 Determination of antioxidative activities

6.3.5.1 DPPH radical scavenging activity

DPPH radical scavenging activity was determined following the method of Khantaphant and Benjakul (2008) (Chapter 2). Sample solution was added with 0.1 mM DPPH in 95% ethanol at a ratio of 1:1 (v/v). The mixture was allowed to stand in dark at room temperature and the absorbance was read at 517 nm after 30 min incubation using UV-1800 Spectrophotometer (Shimadzu, Kyoto, Japan). The control was prepared in the same manner except that distilled water was used instead of the sample. The DPPH radical scavenging activity was calculated from Trolox standard curve (0-60 μM) and expressed as μmol Trolox equivalents (TE)/g protein

6.3.5.2 ABTS radical scavenging activity

ABTS radical scavenging activity was determined according to the method of Re *et al*. (1999). To generate ABTS radical ($ABTS^{\bullet+}$), ABTS stock solution

was mixed with potassium persulfate (2.6 mM) at the ratio of 1:1 (v/v). The mixture was allowed to react in dark for 12 h at room temperature. $ABTS^{\bullet+}$ solution was diluted with methanol at the ratio of 1:50 (v/v). The reaction was initiated by adding 150 µL of sample into 2.85 mL of $ABTS^{\bullet+}$ solution. The mixture was incubated at room temperature for 2 h in dark. The absorbance was then read at 734 nm. Trolox standard curve (0-200 µM) was prepared. For the control, distilled water was used instead of the sample and all assays were carried out in the same manner. ABTS radical scavenging activity was expressed as µmol TE/g protein.

6.3.5.3 Ferric reducing antioxidant power (FRAP)

FRAP was evaluated by the method of Benzie and Strain (1996). A freshly prepared 2.85 ml of FRAP reagent (mixture of 10 mM TPTZ solution in 40 mM HCl, 20 mM $FeCl_3.6H_2O$ solution and 300 mM acetate buffer, pH 3.6 (1:1:10 v/v/v)) was incubated at 37°C for 30 min before mixing with 150 µL of sample. The reaction mixture was allowed to react at room temperature for 30 min in dark. Absorbance at 593 nm was read and FRAP was calculated from the Trolox standard curve (0-60 µM) and expressed as µmol TE/g protein. The control was prepared in the same manner except that distilled water was used instead of the sample.

6.3.5.4 Ferrous chelating activity

Chelating activity towards ferrous ion (Fe^{2+}) was determined by the method of Benjakul *et al*. (2005a) with a slight modification. A 200 µL of sample was mixed with 800 µL of distilled water prior to mixing with 0.1 mL of 2 mM $FeCl_2$ and 0.2 mL of 5 mM ferrozine. The mixture was allowed to stand for 20 min at room temperature. The absorbance was then read at 562 nm. The standard curve of EDTA (0-1.0 mM) and the control were prepared in the same manner except that EDTA, except distilled water was used instead of the sample. Ferrous chelating activity was expressed as µmol EDTA equivalents/g protein.

6.3.6 Isolation and identification of antioxidative peptides

6.3.6.1 A series of column chromatography

HAP or HFP were dissolved in distilled water to obtain the protein concentration of 100 mg/mL. The sample (3 mL) was loaded onto a Sephadex G-25 gel filtration column (2.5 x 50 cm) (Bio-Rad Laboratories, Hercules, CA, USA), pre-equilibrated with distilled water. The elution was performed using distilled water at a flow rate of 0.5 mL/min. Fractions of 3 mL were collected and their absorbances were recorded at 220 and 280 nm. The fractions from HAP and HFP were subjected to analyses for ABTS radical scavenging activity and ferrous chelating activity, respectively. Active fractions with the highest ABTS radical scavenging activity and ferrous chelating activity from HAP and HFP, respectively, were pooled and lyophilized into powder. Lyophilized HAP and HFP were re-dissolved in 50% methanol to obtain the concentration of 100 mg/mL and a 5 uL of sample was further subjected to Phenogel^{TM} gel permeation column (7.8 x 300 mm) (Phenomenex Inc., Torrance, CA, USA), pre-equilibrated with 100% methanol (HPLC grade) at a flow rate of 0.5 mL/min. Fractions of 0.5 mL were collected and subjected to the measurement of A_{220}. The fractions obtained from HAP and HFP were tested for ABTS radical scavenging activity and ferrous chelating activity, respectively.

The fractions with the highest antioxidative activity were then injected into Ultimate 3000 LC System (Dionex Corporation, IL, USA) coupled to HCT Ultra PTM Discovery System ESI-Ion Trap MS (Bruker Daltonics, Germany) with a flow rate of 300 nL/min to a nanocolumn (Acclaim PepMap 100 C18, 3 μm, 100A, 75 μm x 150 mm). A solvent gradient (solvent A: 0.1% formic acid in water; solvent B: 0.1% formic acid in 80% acetonitrile) was run in 30 min.

6.3.6.2 Proteins quantitation and identification

DeCyder MS Differential Analysis Software (DeCyder MS) (GE Healthcare, UK) was used for identification and quantitation of LC-MS data by converting the data and using the PepDetect module for automated peptide detection, charge state assignments, and quantitative comparison based on the peptide ions signal intensities in MS mode. Thereafter, the differential tandem mass spectra of

peptides analyzed by DeCyderMS were identified by the database search software Mascot (Matrix Science, London, UK), the search engine that uses mass spectrometry data to identify proteins from primary sequence databases (Perkins *et al*., 1999). The database was obtained from the NCBI (http://www.ncbi.nlm.gov). All entries taxonomy was chosen and the conditions are fixed at carbamidomethyl (C) and oxidation (M). Mass tolerance for precursor and fragment ions was 1.2 and 0.6 Da, respectively. Other search parameters were monoisotopic mass; peptide charge, 1+ 2+ 3+; instrument, ESI-Trap.

The LC-MS/MS data were then subjected to PEAKS Online *de novo* software to generate amino acid sequences (http://www.bioinfor.com/peaksonline/). The PEAKS Online (Bioinformatics Solutions, Waterloo, Ontario, Canada) *de novo* sequencing software was used for automated *de novo* sequencing. A parent- and fragment-mass error tolerance of 0.08 u; trypsin as the protease with one maximum missed cleavage allowed; deconvolute the charge state in the spectra to generate a spectra in which each monoisotopic peak becomes singly charged; partial modification of cysteine (carbamidomethyl-cysteine) and methionine (oxidized), were used as the *de novo* sequencing parameters. The most abundant peptide fragments *'b-ions* and *y-ions*'; the less abundant peptide fragments '*a-ions*'; the neutral losses of water and ammonia for *b-ions* and *y-ions*; as well as the *immonium ions* were utilized to develop confident and complete peptide sequences *de novo* from MS/MS spectra (Ma *et al*., 2003).

6.3.7 Determination of protein concentration

Protein concentration was measured by the method of Lowry *et al*. (1951) using bovine serum albumin as a standard.

6.3.8 Statistical analysis

Experiments were run in triplicate. All data were subjected to analysis of variance (ANOVA) and differences between means were evaluated by Duncan's

Multiple Range Test (Steel and Torrie, 1980). For pair comparison, T-test was used. SPSS Statistic Program (Version 10.0) (SPSS Inc, Chicago, IL, USA) was used for data analysis.

6.4 Results and Discussion

6.4.1 Antioxidative activities of protein hydrolysate

Protein hydrolysate from the muscle of brownstripe red snapper, HAP and HFP, showed different antioxidative activities when tested by different assays as presented in Table 10. The higher DPPH and ABTS radical scavenging activities and FRAP were observed for HAP ($p < 0.05$), whereas HFP had higher ferrous chelating activity than did HAP ($p < 0.05$). The results suggested that both hydrolysates might scavenge ABTS and DPPH radicals, differently. HAP could react with free radicals to terminate the radical chain reaction more effectively as well as acted as a better reducing compound than did HFP. However, the higher ferrous chelating activity of HFP suggested that HFP might have the peptides with higher capacity of binding ferrous ion. Therefore, both HAP and HFP might have the differences in the amino acid composition, sequence and chain length of peptides. This was governed by the varying specificity of different proteases towards pretreated muscle proteins (Thainsilakul *et al*., 2007a). HAP and HFP were therefore subjected to isolation and identification of antioxidative peptides based on their ABTS radical scavenging activity and ferrous chelating activity.

6.4.2 Separation of antioxidative fractions

Fractionation of antioxidative peptides in HAP and HFP using Sephadex G-25 gel filtration was carried out as shown in Figure 30 and 31, respectively. Fractions were collected and the absorbances at 220 and 280 nm and antioxidative activity were monitored. The absorbance at 220 nm (A_{220}) represents peptide bond, whereas the absorbance at 280 nm (A_{280}) indicates proteins, peptides or

amino acids with aromatic ring such as tyrosine and tryptophan (Amarowicz and Shahidi, 1997).

Table 10. Antioxidative activities of protein hydrolysate from the muscle of brownstripe red snapper prepared using Alcalase/pyloric caeca protease (PCP) (HAP) and Flavourzyme/PCP (HFP)

Antioxidative activities	HAP	HFP
DPPH radical scavenging activity*	$19.44 \pm 0.03^{a\dagger}$	14.90 ± 0.49^{b}
ABTS radical scavenging activity*	1302.65 ± 5.31^{a}	1057.00 ± 9.21^{b}
Ferric reducing antioxidant power*	16.02 ± 0.01^{a}	12.70 ± 0.13^{b}
Ferrous chelating activity**	20.33 ± 0.42^{b}	49.27 ± 0.51^{a}

Means ± SD (n=3)
†Different subscripts in the same row indicate the significant differences ($p < 0.05$).
*μmol Trolox/g protein
**μmol EDTA/g protein

In general, peptide containing tyrosine usually exhibited a strong free radical scavenging activity due to the phenolic hydroxyl groups of tyrosine residue, which contribute substantially for scavenging of free radicals via the mechanism of donating a hydrogen atom from their hydroxyl group (Suetsuna *et al*., 2000; Guo *et al*., 2009). Other aromatic amino acids, tryptophan and phenylalanine, are generally considered as effective radical scavengers, because they can donate protons easily to electron deficient radicals while at the same time maintaining their stability via resonance structures (Rajapakse *et al*., 2005; Zhang *et al*., 2009). The higher DPPH and ABTS radical scavenging activity and FRAP of HAP was probably due to the higher content of these aromatic amino acids. When ABTS radical scavenging activity of all fraction of HAP was determined (Figure 30B), one major active peak with the highest activity was found. Active fractions (fraction no. 43-44) were pooled and concentrated by lyophilization for further isolation. For HFP, however two activity peaks were observed (Figure 31B), the fractions from the second peak showing the higher chelating activity (fractions no. 46-47) were pooled and lyophilized for further isolation.

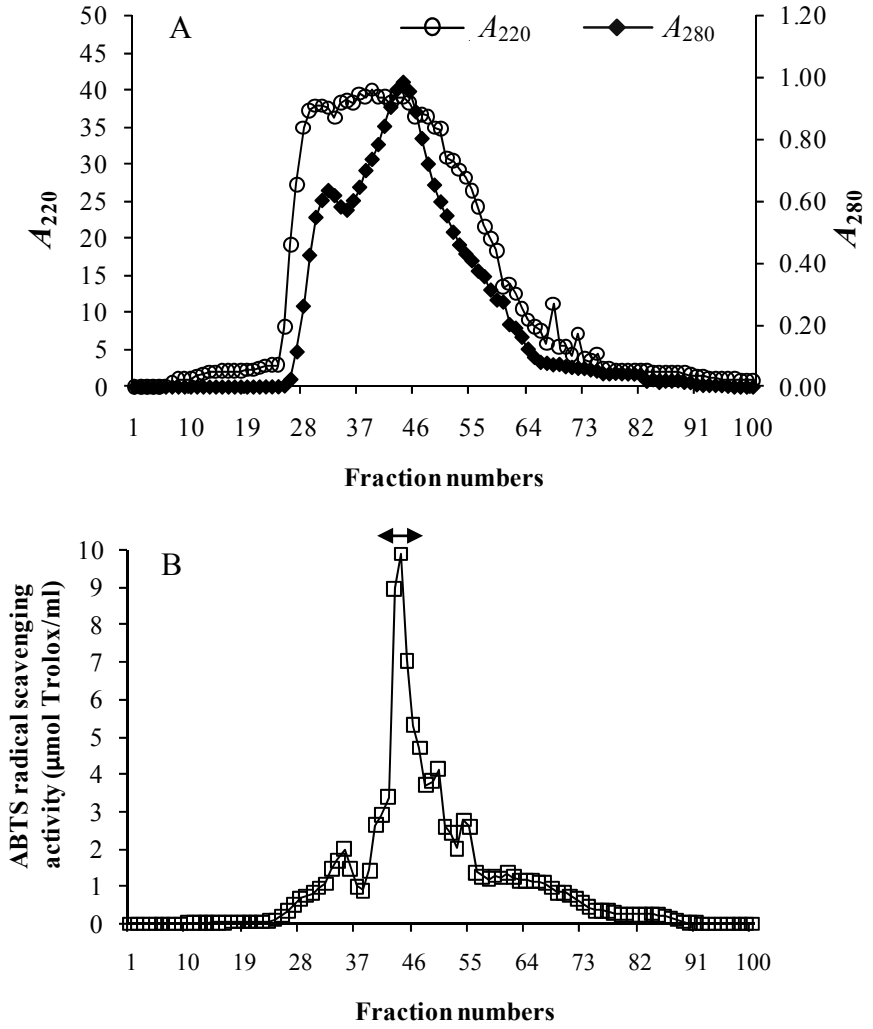

Figure 30. Elution profile of HAP from Sephadex G-25 column monitoring by A_{220} and A_{280} (A). Elution was performed using distilled water with a flow rate of 0.5 mL/min. Fractions (3 mL) were determined for ABTS radical scavenging activity (B). (◄►): pooled fractions.

Lyophilized Sephadex fraction of HAP with the high ABTS radical scavenging activity and that of HFP with the high ferrous chelating activity were re-dissolved in 50% methanol to obtain final concentration of 100 mg protein/mL and subjected to Phenogel^{TM} column. The elution profiles of Sephadex fraction of HAP and HFP from Phenogel^{TM} column are depicted in Figure 32(a) and (b), respectively.

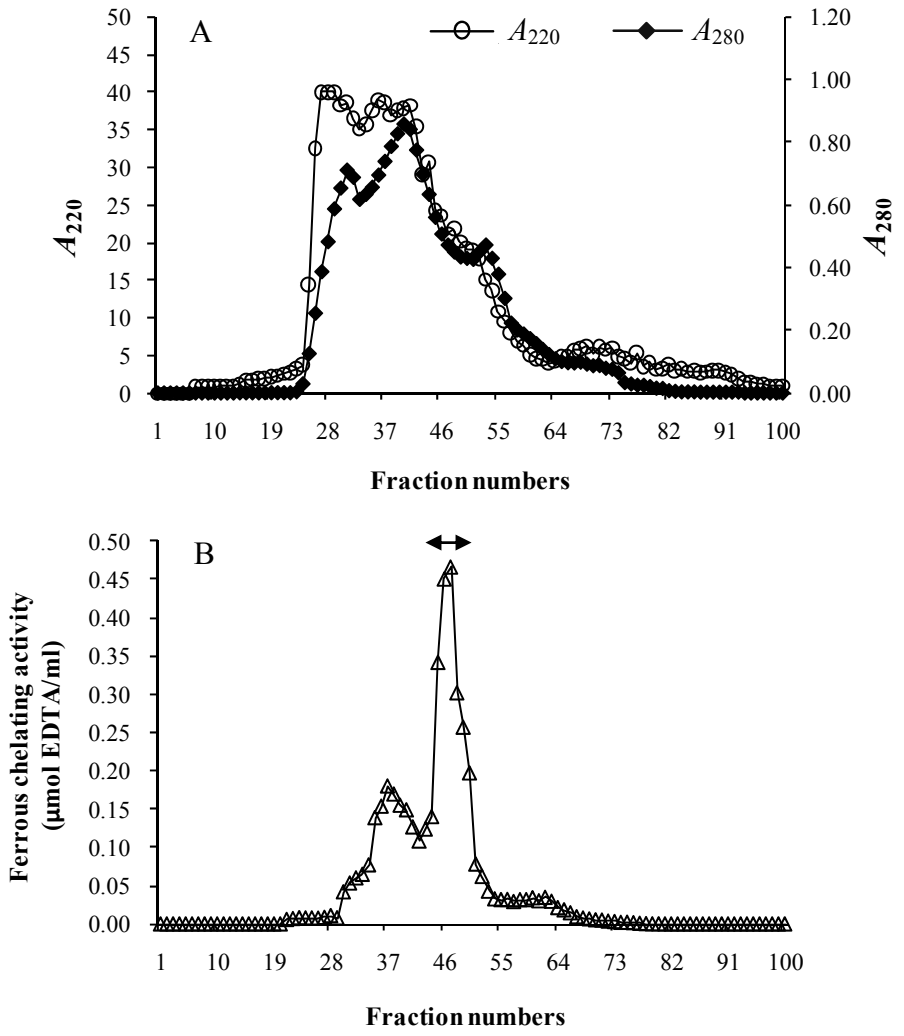

Figure 31. Elution profile of HFP from Sephadex G-25 column monitoring by A_{220} and A_{280} (A). Elution was performed using distilled water with a flow rate of 0.5 mL/min. Fractions (3 mL) were determined for ABTS radical scavenging activity (B). (↔): pooled fractions.

After elution, only one active peak was observed for Phenogel^{TM} fractions of both hydrolysates. The active fractions were pooled and the methanol was eliminated by evaporation. Thereafter the Phenogel^{TM} fractions were subsequently subjected to LC-MS for determination of peptide mass and identified for peptide sequence using DeCyder MS and PEAKS online softwares.

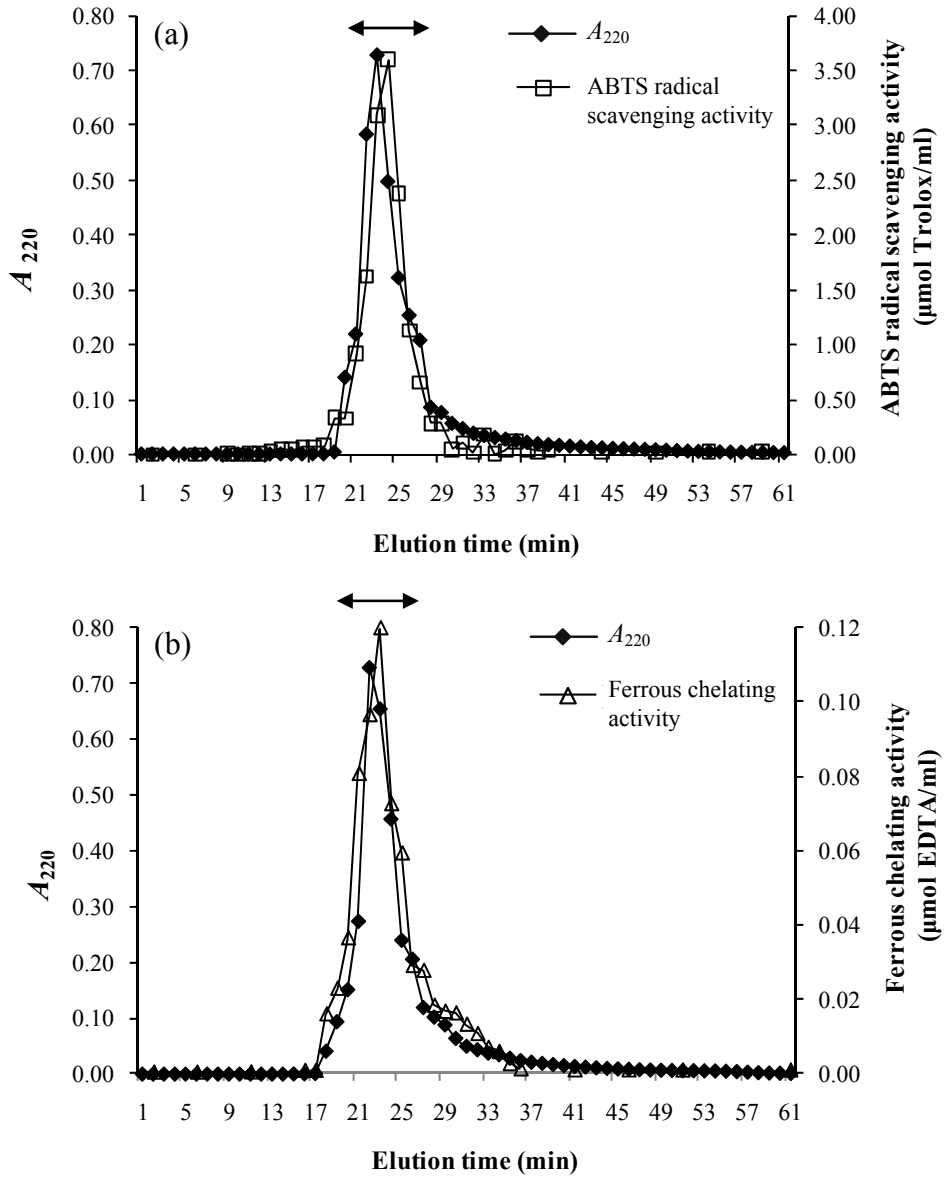

Figure 32. Elution profile of Sephadex fraction of HAP (a) and HFP (b) from Phenogel™ column monitoring by A_{220} and different antioxidative activities. Elution was performed using 100% methanol with a flow rate of 0.5 mL/min. Fractions (0.5 mL) were determined for ABTS radical scavenging activity for HAP and ferrous chelating activity for HFP. (↔): pooled fractions.

The purification steps of HAP and HFP are shown in Table 11 and 12, respectively. The chromatography techniques were shown to increase the purity of antioxidative peptides from both hydrolysates. After isolation on Sephadex G-25, the purity of 13.9-fold was observed for HAP fractions with recovery yield of 21.7%. HFP fractions had the 14.4-fold higher in purity after Sephadex G-25 chromatography with the lower yield (18.7%), compared with that of HAP fractions. Phenogel^{TM} column increased the purity of HAP fractions up to 38.0-fold, comparing to that of crude HAP. The yield of 15.5% was obtained. HFP fractions from Phenogel^{TM} column had 28.4-fold increase in purity with 11.4% yield. The increase in purity was owing to the removal of contaminating peptides after chromatographies. A series of chromatographies has been used successfully to isolate and purify peptides. Purified peptides from Alaska pollack frame protein hydrolysate with antioxidative activity were obtained after consecutive chromatographic technique on SP-Sephadex C-25 ion exchange column, Sephadex G-75 gel filtration column and reversed-phase high performance liquid chromatography (RP-HPLC), respectively (Je *et al*., 2005). Antioxidative peptide from hoki frame protein hydrolysate was purified using ion exchange chromatography followed by reverse-phase HPLC (Kim *et al*., 2007). Moreover, purified peptides derived from hydrolysates of sardinelle (*Sardinella aurita*) by-products proteins were fractionated on a Sephadex G-25 gel filtration column followed by RP-HPLC (Bougatef *et al*., 2010b).

6.4.3 Identification of antioxidative peptides by LC-MS

To identify the antioxidative peptides, methanol free sample eluted from Phenogel^{TM} column was injected into LC system coupled on-line to ion trap MS. The LC-MS data was obtained and subjected to PEAKS Online *de novo* sequencing software to generate amino acid sequences. The sequences of peptides were determined as shown in Table 13.

Table 11. Purification step of antioxidative peptides with ABTS radical scavenging activity from protein hydrolysate prepared using Alcalase/PCP (HAP) by a series of chromatographies.

Purification step	Total activity (μmol Trolox)	Total protein (mg protein)	Specific activity (μmol Trolox/mg protein)	Purity (fold)	Yield (%)
Crude HAP	357.65	300	1.19	1.0	100.0
Sephadex G-25	77.70	4.68	16.60	13.9	21.7
Phenogel™	55.39	1.22	45.24	38.0	15.5

Table 12. Purification step of antioxidative peptides with ferrous chelating activity from protein hydrolysate prepared using Flavourzyme/PCP (HFP) by a series of chromatographies.

Purification step	Total activity (μmol EDTA)	Total protein (mg protein)	Specific activity (μmol EDTA/mg protein)	Purity (fold)	Yield (%)
Crude HFP	14.66	300	0.05	1.0	100.0
Sephadex G-25	2.75	3.91	0.70	14.4	18.7
Phenogel™	1.67	1.21	1.39	28.4	11.4

Table 13. Amino acid sequences of antioxidative peptides derived from brownstripe red snapper protein hydrolysate

Samples	Antioxidative peptides	Mass (Da)
HAP	Asn-Arg-Lys-Arg	574
	Asp-Ala-Gly-Leu-Phe-Lys	650
	Met-Glu-Glu-Asp-Leu-Glu-Glu-Arg	1,051
	Met-Ser-Leu-Trp-Gln-Ser-Leu-Met-Asn-Asp-Lys	1,353
HFP	Cys-Gly-Asp-Ser-Val-Lys	609
	Met-Cys-Cys-Cys-Arg	615
	His-Arg-Arg-Arg	625
	Asn-Phe-Cys-Ser-Arg	626
	Trp-Trp-Arg-Lys	676
	Phe-Cys-Gly-Val-Ala-Thr-Lys	726

Generally, bioactive peptides usually consist of 3-20 amino acid residues (Je *et al*., 2007) and their activity is based on their amino acid composition and sequence. Identified peptides from HAP contained 4-11 amino acid residues, whereas the shorter chain with 4-7 amino acid residues were identified from antioxidative peptides of HFP. Klompong *et al*. (2007) suggested that size of peptides influences the antioxidative activity. Mostly, the lower molecular weight peptides possessed higher antioxidative activity. The higher lipid oxidation inhibition of protein hydrolysate from giant squid (*Dosidicus gigas*) was found for low MW peptides (< 3 kDa) than that of the higher ones (Rajapakse *et al*., 2005). Kim *et al*. (2009) reported that purified peptide from venison protein hydrolysate with MW of 9,853 Da exhibited the stronger antioxidative activity than did the 11,213 Da peptide. However, other reports suggested the negative activity of small peptide such as the peptide from mackerel hydrolysate, in which the 900 and 200 Da peptides showed the lower antioxidative activity than did the 1,400 Da peptide (Wu *et al*., 2003).

Type of amino acid in peptides also affected the antioxidative activity of resulting peptides. Kaur and Kapoor (2001) suggested that amino acids are one of the natural antioxidants that may act as singlet oxygen quenchers or chain breaking antioxidants. Peptides with ABTS radical scavenging activity might contain amino acid with capacity of scavenging the free radical or providing the electron to the radicals, especially, aromatic amino acids. Apart from aromatic amino acids, tyrosine (Tyr), tryptophan (Trp) and phenylalanine (Phe), cysteine (Cys) are able to donate the sulfur hydrogen (Hernández-Ledesma *et al*., 2005), methionine (Met) and lysine (Lys) have been also reported to possess antioxidative activity (Kim *et al*., 2001; Park *et al*., 2001). Furthermore, hydrophobic peptides can help scavenging of free radicals by keeping close contact with oxidizing substance, leading to the rapid scavenging of radicals (Mendis *et al*., 2005a; 2005b). Suetsuna *et al*. (2000) indicated that some hydrophobic amino acid such as proline (Pro), alanine (Ala) and leucine (Leu) contributed to scavenging of free radicals. Some reports suggested the high antioxidative activities resulted from the presence of acidic amino acid such as glutamic acid (Glu) and aspartic acid (Asp) in the peptide chains (Saiga *et al*., 2003). Additionally, arginine (Arg), a diamine monocarboxylic amino acid with a guanidinium group, has been confirmed to have the antioxidative effect (Lass *et al*., 2002; Lin *et al*., 2005).

All identified peptides derived from HAP contained amino acids possessing as antioxidant, especially peptide with Met-Glu-Glu-Asp-Leu-Glu-Glu-Arg in sequence, in which all amino acids were recognized to possess antioxidative activities. Identified peptides from HFP also contained the antioxidative amino acids in the sequences. Three identified peptides of six peptides (Met-Cys-Cys-Cys-Arg, His-Arg-Arg-Arg and Trp-Trp-Arg-Lys) consisted of antioxidative amino acids in their sequences. Kim *et al*. (2001), Wu *et al*. (2003) and Je *et al*. (2005) reported that the noticeable chelating activity of histidine-containing peptides was attributed to the proton-donation ability of imidazole group. In addition, the carboxylic and amino groups in branches of the acidic (Asp and Glu) and basic (Arg, Lys and His) amino acids were able to enhance Fe^{2+} chelating of resulting peptides (Liu *et al*., 2010). Therefore, ferrous chelating peptides, especially His-Arg-Arg-Arg, might play a key

role for obviously ferrous chelating activity of HFP. It was noted that the carboxyl terminus (C-terminus) amino acids of all identified peptides were lysine or arginine, which most likely resulted from the specificity of PCP used in the second step of hydrolysis process. PCP from brownstripe red snapper has been classified as trypsin-like enzyme (Khantaphant and Benjakul, 2010) (Chapter 3). Trypsin is a pancreatic serine protease, which specifically hydrolyzes peptide bonds at the C-terminus of lysine and arginine residues. The specificity of PCP toward amino acids was advantageous, in which lysine and arginine could be localized at C-terminus with enhanced antioxidative activity.

Moreover, the sequence of amino acid in peptide chain has an impact on antioxidative activity. Peptides containing tyrosine residues at the C-terminus, lysine or phenylalanine residues at the N-terminus were reported to have strong free radical scavenging activity (Guo *et al*., 2009). Leucine and proline residues could favor antioxidant activity once it present in the C-terminus end of the sequence (Mendis *et al*., 2005b; Suetsuna *et al*., 2000). However, some report suggested the high antioxidative activities resulted from the presence of hydrophobic amino acid residue, valine or leucine at the N-terminus (Chen *et al*., 1998). Only Phe-Cys-Gly-Val-Ala-Thr-Lys from HFP that consisted of phenyalanine at N-terminus, thus the antioxiative activity of this peptide might result from the presence of mentioned amino acid residue. In conclusion, the different amino acids in terms of type, sequence and chain length of active peptides from both HAP and HFP resulting in the different antioxidative activities.

6.5 Conclusion

Protein hydrolysates from the muscle of brownstripe red snapper prepared by two-step hydrolysis process using Alcalase or Flavourzyme in the first step of hydrolysis followed by pyloric caeca protease (PCP) from brownstripe red snapper in the second step (HAP and HFP, respectively) showed the different antioxidative activities. HAP exhibited the greater radical scavenging activities and FRAP, whereas HFP showed the remarkable ferrous chelating activity. Sephadex G-

25 gel filtration and Phenogel™ gel permeation chromatographies resulted in the higher purity of antioxidative peptide isolated. Radical scavenging peptides from HAP include Asn-Arg-Lys-Arg, Asp-Ala-Gly-Leu-Phe-Lys, Met-Glu-Glu-Asp-Leu-Glu-Glu-Arg and Met-Ser-Leu-Trp-Gln-Ser-Leu-Met-Asn-Asp-Lys. Ferrous chelating peptides from HFP were Cys-Gly-Asp-Ser-Val-Lys, Met-Cys-Cys-Cys-Arg, His-Arg-Arg-Arg, Asn-Phe-Cys-Ser-Arg, Trp-Trp-Arg-Lys and Phe-Cys-Gly-Val-Ala-Thr-Lys. Therefore, protein hydrolysate from brownstripe red snapper can be a potential source of antioxidative peptides, which can be further used as a natural antioxidant for food application or functional foods.

CHAPTER 7

CHARACTERIZATION OF PROTEIN HYDROLYSATES FROM THE MUSCLE OF BROWNSTRIPE RED SNAPPER: THEIR ANTIOXIDATIVE ACTIVITIES, FUNCTIONAL PROPERTIES, STORAGE STABILITY AND THE FORTIFICATION IN DRINK

7.1 Abstract

Antioxidative activities, functional properties and storage stability of protein hydrolysates from the muscle of brownstripe red snapper (*Lutjanus vitta*) prepared by two-step hydrolysis process were investigated. Both hydrolysate prepared using Alcalase followed by pyloric caeca protease (PCP) (HAP) and that prepared by Flavourzyme followed by PCP (HFP) contained high protein content (87.36 and 86.55% for HAP and HFP, respectively). Both HAP and HFP contained glutamic acid/glutamine as the major amino acids, followed by aspartic acid/asparagine, lysine, alanine and leucine, respectively. Conversely, both hydrolysates contained low levels of cysteine and tryptophan. HAP showed the higher DPPH and ABTS radical scavenging activities and ferric reducing antioxidant power (FRAP), compared with HFP ($p < 0.05$). Nevertheless, higher ferrous chelating activity was observed in HFP ($p < 0.05$). Antioxidative activities of both hydrolysates were stable over a wide temperature range (25-100°C), except for DPPH radical scavenging activity of HFP that slightly decreased when temperature was in the range of 50-100°C. When heated at 100°C up to 120 min, the antioxidative stabilities of both hydrolysates were observed, except for ferrous chelating activities that showed the slight decreases when heating time was longer than 60 min. High stability over a wide pH range (2-12) was also observed for both HAP and HFP. However, ABTS radical scavenging activity slightly decreased in very alkaline pH ranges ($p < 0.05$). HAP and HFP were soluble over a wide pH range (2-12) with solubility higher than 98%. Both HAP and HFP

exhibited interfacial properties in dose-dependent manner. HFP had the higher emulsifying and foaming properties than did HAP (p <0.05). During storage at 4°C for 12 weeks, the antioxidative activities of HAP and HFP slightly decreased with coincidental increase in yellowness (b* value) (p <0.05). Fortification of freshly prepared or 8-week stored HAP and HFP at levels of 0.3 and 0.4% in soybean milk enhanced the antioxidative activities with no differences in overall likeness, compared with the original soybean milk. Therefore, protein hydrolysate from brownstripe red snapper could be used as an alternative antioxidant as well as functional foods.

7.2 Introduction

Fish have been increasingly demanded among the consumers due to the high nutritive value (Guerard *et al*., 2001). Apart from being processed to a variety of products for human consumption, fish can be produced as active ingredient or functional food with bioactivities. Fish protein hydrolysates containing active peptides have been paid increasing attention. Protein hydrolysates have been reported as the suitable sources of protein for human nutrition. Short chain peptides could be of ease for the gastrointestinal absorption (Clemente *et al*., 2000). Many dietary proteins from marine sources possess specific biological properties after proteolytic hydrolysis using various proteases (Amarowicz and Shahidi, 1997; Kristinsson and Rasco, 2000b; Wu *et al*., 2003; Sathivel *et al*., 2003; Klompong *et al*., 2007; Thiansilakul *et al*., 2007a; Qian *et al*., 2008a; 2008b). Adler-Nissen (1979) suggested that the different antioxidative activities were governed by protease specificity, nature of protein substrate and degree of hydrolysis. In general, protein hydrolysates, especially fish protein hydrolysates from enzymatic hydrolysis, are the best among protein hydrolysates, in term of nutritional properties, balanced amino acid composition and high digestibility (Kristensson and Rasco, 2000b). Furthermore, proteins cleaved into smaller molecules or peptides during enzymatic hydrolysis process could improve and upgrade the functional and nutritional properties of food proteins (Kudo *et al*., 2009). Hydrolysis process therefore has been developed to convert such resources into marketable and acceptable forms (Gildberg, 1993). Fish protein hydrolysates can

served as a potential food supplements (Venugopal and Shahidi, 1995). In some countries, fish protein hydrolysates are used as a milk substitute and as flavoring compounds (Stephens *et al*., 1976).

Functional properties influence the use of protein hydrolysate as an ingredient in food and also provide the physical properties during processing and storage (Sathivel *et al*., 2005). Controlled hydrolysis can provide and improve functional properties of protein (Klompong, *et al*., 2007). Hydrolysis process directly influences the molecular size, hydrophobicity and polar group of the hydrolysate (Kristensson and Rasco, 2000b). Functional properties of protein including interfacial properties were affected directly by characteristic of protein hydrolysates (Klompong *et al*., 2007). Gbogouri *et al*. (2004) indicated that protein hydrolysate has an excellent solubility at high degree of hydrolysis. High solubility of fish protein hydrolysate over a wide pH range is an advantageous characteristic to be applied in many foods (Klompong *et al*., 2007).

In Thailand, brownstripe red snapper (*Lutjanus vitta*) is one of the raw materials for surimi production (Khantaphant and Benjakul, 2010) (Chapter 3). Production of fish protein hydrolysates with antioxidant activity can pave the way for full utilization of such species. Moreover, its viscera, especially pyloric ceaca, generated during processing, can be used as the promising source of proteases (Khantaphant and Benjakul, 2008) (Chapter 2). The use of protease from this source in conjunction with other commercially available proteases may lead to the lower cost of enzyme as well as to the novel peptides with pronounced antioxidative activity. This work aimed to study functional properties and antioxidative activities of protein hydrolysate from brownstripe red snapper muscle prepared by two-step hydrolysis process using Alcalase or Flavourzyme in the first step of hydrolysis, followed by pyloric caeca protease (PCP) from brownstripe red snapper for the second step. Moreover, the stability during extended storage was investigated and the fortification of hydrolysate in drink was also tested.

7.3 Materials and Methods

7.3.1 Chemicals

Alcalase 2.4 L (E.C. 3.4.21.62) (2.4 AU/g) and Flavourzyme 500 L (E.C. 3.4.21.77) (500 LAPU/g) were provided by Novozyme (Bagsvaerd, Denmark). 1,1-diphenyl-2-picrylhydrazyl (DPPH), 3-(2-pyridyl)-5,6-diphenyl-1,2,4-triazine-4′,4″-disulfonic acid (ferrozine), 2, 4, 6-tripyridyl-triazine (TPTZ), 2,2′-azinobis(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS) and sodium dodecylsulphate (SDS) were obtained from Sigma (St. Louis, MO, USA). Thiobarbituric acid (TBA) and potassium persulfate were purchased from Fluka (Buchs, Switzerland). Sodium sulfite was procured from Riedel-deHaën (Seelze, Germany). All chemicals were of analytical grade.

7.3.2 Preparation of pretreated fish mince

Brownstripe red snapper, off-loaded approximately 24-36 h after capture, were purchased from a dock in Songkhla province, Thailand. Fish were transported in ice with the fish/ice ratio of 1:2 (w/w) to the Department of Food Technology, Prince of Songkla University within 1 h. Upon arrival, whole fish were washed and only flesh was separated manually. Flesh was minced to uniformity using Moulinex AY46 blender (Group SEB, Lyon, France) before homogenizing with nine volumes of cold 8 mM $CaCl_2$ solution containing 5 mM citric acid using an IKA Labortechnik homogenizer (Selangor, Malaysia) at a speed of 11,000 rpm for 2 min. After continuous stirring for 60 min at 4°C, the sample was centrifuged at 4,000 x *g* for 15 min at 4°C using a Beckman Coulter centrifuge Model Avant J-E (Beckman Coulter, Inc., Fullerton, CA, USA). Thereafter, the pellet was washed by homogenizing with five volumes of cold distilled water using a homogenizer at a speed of 11,000 rpm for 2 min, followed by stirring at 4°C for 15 min prior to centrifuging at 9,600 x *g* for 10 min at 4°C. The washing process was repeated twice.

Pretreated mince obtained was kept in polyethylene bags and placed in ice until use, but not longer than 2 h.

7.3.3 Preparation of proteases from pyloric caeca

Pyloric caeca from brownstripe red snapper was collected and powdered in liquid nitrogen. Thereafter, the pyloric caeca extract was prepared according to the method of Khantaphant and Benjakul (2010) (Chapter 3). Pyloric caeca powder was suspended in ten volumes of 50 mM Tris-HCl buffer, pH 8.0, containing 10 mM $CaCl_2$. The mixture was homogenized at 11,000 rpm for 2 min. The homogenate was continuously stirred for 30 min at 4°C and centrifuged at 8000 x *g* for 30 min at 4°C. The supernatant was filtered through a Whatman filter paper No. 1 (Whatman International Ltd., Maidstone, England). The filtrate obtained was further subjected to 40-60% saturation ammonium sulfate precipitation. After stirring at 4°C for 30 min, the mixture was centrifuged at 8,000 x *g* for 30 min at 4°C and the pellet obtained was dissolved in 50 mM Tris-HCl buffer, pH 8.0 followed by dialysis 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 'pyloric caeca protease; PCP'. PCP was determined for proteolytic activity using casein as a substrate under optimal condition (60°C and pH 8.5).

7.3.4 Production of brownstripe red snapper protein hydrolysates

Pretreated brownstripe red snapper mince (95.42% protein dry basis) was mixed with 50 mM Tris-HCl buffer, pH 7.0 and 8.0 for hydrolysis using Flavourzyme and Alcalase, respectively, to obtain a final protein concentration of 2% (w/v). The mixtures were homogenized at a speed of 11,000 rpm for 1 min and the homogenates were pre-incubated at 50°C for 10 min. The hydrolysis reaction was initiated by adding Alcalase or Flavourzyme at the amount required to gain DH of 40% following the method of Benjakul and Morrissey (1997). After 2 h of hydrolysis, the reactions were inactivated by heating the mixture in boiling water for 10 min. The

mixture was then centrifuged at 2,000 x *g* at 4°C for 10 min and the supernatant was collected and referred to as protein hydrolysate prepared using Alcalase (HA) and Flavourzyme (HF). The further hydrolysis by PCP from brownstripe red snapper was conducted at pH 8.5 and 60°C for 1 and 2 h for HF and HA, respectively. The hydrolysis was finally stopped by placing the mixture in boiling water for 10 min followed by centrifugation at 2,000 x *g* at 4°C for 10 min. The supernatant was collected and lyophilized. The lyophilized brownstripe red snapper protein hydrolysates prepared using Alcalase/PCP and Flavourzyme/PCP were referred to as 'HAP' and 'HFP', respectively. The obtained hydrolysates were subjected to analyses.

7.3.5 Proximate analysis

HAP and HFP were determined for protein, fat, ash and moisture contents according to the methods of AOAC (2000) with the analytical No. of 992.15, 991.36, 942.05 and 950.46, respectively. Protein, fat and ash contents were expressed on a dry weight basis.

7.3.6 Amino acid analysis

HAP and HFP were hydrolyzed under reduced pressure in 4.0 M methanesulfonic acid containing 0.2% (v/v) 3-2(2-aminoethyl) indole at 115 °C for 24 h. The hydrolysates were neutralized with 3.5 M NaOH and diluted with 0.2 M citrate buffer (pH 2.2). An aliquot of 0.4 mL was applied to an amino acid analyzer (MLC-703; Atto Co., Tokyo, Japan).

7.3.7 Determination of antioxidative activities and stability

7.3.7.1 Antioxidative activity

- DPPH radical scavenging activity

DPPH radical scavenging activity was determined following the method of Khantaphant and Benjakul (2008) (Chapter 2). Sample solution was added with 0.1 mM DPPH (in 95% ethanol) at a ratio of 1:1 (v/v). The mixture was allowed to stand in dark at room temperature and the absorbance was read at 517 nm after 30 min incubation using UV-1800 Spectrophotometer (Shimadzu, Kyoto, Japan). The control was prepared in the same manner except that distilled water was used instead of the sample. The DPPH radical scavenging activity was calculated from Trolox standard curve (0-60 μM) and expressed as μmol Trolox equivalents (TE) / g protein.

- ABTS radical scavenging activity

ABTS radical scavenging activity was determined according to the method of Re *et al*. (1999). To generate ABTS radical ($ABTS^{\bullet+}$), ABTS stock solution was mixed with potassium persulfate (2.6 mM) at the ratio of 1:1 (v/v). The mixture was allowed to react in dark for 12 h at room temperature. $ABTS^{\bullet+}$ solution was diluted with methanol at the ratio of 1:50 (v/v). The reaction was initiated by adding 150 μL of sample into 2.85 mL of $ABTS^{\bullet+}$ solution. The mixture was incubated at room temperature for 2 h in dark. The absorbance was then read at 734 nm. Trolox standard curve (0-200 μM) was prepared. Distilled water was used instead of the sample and prepared in the same manner to obtain the control. ABTS radical scavenging activity was expressed as μmol TE / g protein.

- Ferric reducing antioxidant power (FRAP)

FRAP was evaluated by the method of Benzie and Strain (1996). A freshly prepared 2.85 mL of FRAP reagent (mixture of 10 mM TPTZ solution in 40 mM HCl, 20 mM $FeCl_3.6H_2O$ solution and 300 mM acetate buffer, pH 3.6 (1:1:10 v/v/v)) was incubated at 37°C for 30 min before mixing with 150 μL of sample. The reaction mixture was allowed to react at room temperature for 30 min in dark.

Absorbance at 593 nm was read and FRAP was calculated from the Trolox standard curve (0-60 μM) and expressed as μmol TE / g protein. The control was prepared in the same manner except that distilled water was used instead of the sample.

- Ferrous chelating activity

Chelating activity towards ferrous ion (Fe^{2+}) was determined by the method of Benjakul *et al*. (2005a) with a slight modification. A 200 μL of sample was mixed with 800 μL of distilled water prior to mixing with 0.1 mL of 2 mM $FeCl_2$ and 0.2 mL of 5 mM ferrozine. The mixture was allowed to stand for 20 min at room temperature. The absorbance was then read at 562 nm. The standard curve of EDTA (0-1.0 mM) was prepared. The control was prepared in the same manner except that the distilled water was used instead of the sample. Ferrous chelating activity was expressed as μmol EDTA equivalents / g protein.

7.3.7.2 Antioxidative stability

- Thermal stability

Protein hydrolysates dissolved in distilled water with 2% protein content were incubated at 25, 37, 45, 50, 60, 70, 80, 90 and 100°C in the temperature controlled-water bath (Memmert Model WB 14, Schwabach, Germany) for 30 min. The treated samples were suddenly cooled in iced water. The remaining antioxidative activities were determined. To study the effect of heating time on the remaining activity, the hydrolysate solutions were heated at 100°C for different times (60, 90 and 120 min). After cooling down, the residual antioxidative activity was determined.

- pH stability

Protein hydrolysates were dispersed in distilled water previously adjusted to different pHs (2, 3, 4, 5, 6, 7, 8, 9, 10, 11 and 12) using 1 and 6 N HCl or 1 and 6 N NaOH. The final volume was made up to designated volume to obtain the final protein concentration of 2%. The mixture was allowed to stand at room temperature for 30 min. Thereafter, the pHs of the sample were adjusted to pH 7.0 and the residual antioxidative activities were determined.

7.3.8 Determination of functional properties

7.3.8.1 Solubility

Protein solubility of hydrolysates was determined as described by Klompong *et al*. (2007). Protein hydrolysates were dispersed in distilled water to obtain the final protein concentration of 1% (w/v). The pH of the sample was adjusted to 2, 3, 4, 5, 6, 7, 8, 9, 10, 11 and 12 with 1 and 6 N HCl or 1 and 6 N NaOH. The mixture was stirred at room temperature for 30 min and centrifuged at 7500 x *g* for 15 min. The supernatant was determined for protein content by the Biuret method. Total protein content was determined after solubilization of the sample in 0.5 N NaOH. Protein solubility was calculated as follows:

Solubility (%) = (A/B) x 100

where A is protein content in supernatant and B is total protein content in the sample.

7.3.8.2 Emulsifying properties

Emulsifying properties were determined according to the method of Pearce and Kinsella (1978). Soybean oil and 1% protein hydrolysate solution were mixed at a ratio of 1:3 (v/v). The mixture was homogenized using an IKA homogenizer at a speed of 20000 rpm for 1 min. A 50 µL of emulsion was pipetted at 0 and 10 min and 100-fold dilution with 0.1% SDS solution was made. The absorbance of the diluted solution was measured immediately (A_0) and 10 min after emulsification (A_{10}) at 500 nm. The emulsifying activity index (*EAI*) and the emulsion stability index (*ESI*) were calculated as follows:

EAI (m^2/g) = (2 x 2.303 x A_{500} x DF) / $l\emptyset C$

where A_{500} = absorbance at 500 nm, DF = dilution factor (100), l = path length of cuvette (m), $\emptyset$ = oil volume fraction and C = protein concentration in aqueous phase (g/m^3)

ESI (min) = A_0 x Δt / ΔA

where $\Delta A = A_0 - A_{10}$ and Δt = 10 min

7.3.8.3 Foaming properties

Protein hydrolysates were determined for their foaming properties according to the method of Klompong *et al*. (2007) with a slight modification. A 20 mL of 0.5 and 1% protein hydrolysate solution was homogenized in a 50-mL cylinder at a speed of 16000 rpm, using a homogenizer for 2 min at room temperature. The total volume was read at 30 s after whipping. The foaming capacity was calculated according to the following equation:

Foaming capacity (%) = A/B x 100

where A is the volume after whipping (mL) and B is the volume before whipping (mL). The whipped sample was allowed to stand at room temperature for 10 min and the volume of whipped sample was then read. Foam stability was calculated as follows:

Foam stability (%) = C/A x 100

where C is the volume after standing (mL) and A is the volume after whipping (mL).

7.3.9 Storage stability of protein hydrolysate

Lyophilized HAP and HFP were placed in the tightly closed plastic container and stored at 4°C for 0, 2, 4, 8 and 12 weeks. The samples were taken for determination of antioxidative activities. Thiobarbituric acid reactive substances (TBARS) and color of both hydrolysates were also monitored during the storage.

7.3.9.1 Determination of thiobarbituric acid reactive substances (TBARS)

Thiobarbituric acid reactive substances (TBARS) were determined following the method of Benjakul *et al*. (2005a) with a slight modification. Sample (1 g) was mixed with 4 mL of solution containing 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 x *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. TBARS was calculated from a standard curve of malonaldehyde (MDA) and expressed as mg MDA/kg sample.

7.3.9.2 Colour measurement

The color of HAP and HFP was measured by colorimeter (ColourFlex, HunterLab Reston, VA, USA) and reported in CIE system. *L**, *a** and *b** parameters indicate lightness, redness/greenness and yellowness/blueness, respectively.

7.3.10 Fortification of hydrolysates in soybean milk

7.3.10.1 Preparation of soybean milk fortified with protein hydrolysate

Soybean milk (Vitamilk) (Greenspot Co., Ltd., Thailand) was purchased from a local supermarket, Hat Yai, Thailand. Freshly prepared or 8-week stored HAP and HFP were then added to the soybean milk at different levels (0.1, 0.2, 0.3% for HAP and 0.3, 0.4, 0.5% for HFP). The resulting drinks were then subjected to analyses.

7.3.10.2 Analyses

Prepared soybean milk was determined for antioxidative activity as previously described. Viscosity of samples was determined using a viscometer (DV-II+, Brookfield, MA, USA) at a constant temperature of 25ºC. Spindle No.4 and a speed of 135 rpm were used. Color of drink was also measured as mentioned above. To evaluate the sensory property, hedonic 9-point scale was used (9 = Like extremely,

5 = Neither like nor dislike, 1 = Dislike extremely). Soybean milks without and with the fortification of protein hydrolysates were assessed by thirty panelists with the ages of 22-30 for color, odor, flavor, taste and overall likeness.

7.3.11 Statistical analysis

Experiments were run in triplicate. Analysis of variance (ANOVA) was performed and differences between means were run by Duncan's Multiple Range Test. For pair comparison, T-test was used (Steel and Torrie, 1980). SPSS Statistic Program (SPSS 10.0 for Windows, SPSS Inc, Chicago, IL, USA) was used for data analysis.

7.4 Results and Discussion

7.4.1 Proximate compositions of protein hydrolysates

Chemical compositions of protein hydrolysate from the muscle of brownstripe red snapper prepared using Alcalase/PCP (HAP) and Flavourzyme/PCP (HFP) are shown in Table 14. HAP and HFP derived from the muscle of brownstripe red snapper with the yield of 15.15 and 14.89% (based on wet weight of fish muscle) had high protein content (87.36 and 86.55% for HAP and HFP, respectively). This suggested that both HAP and HFP were the essential source of proteins. During hydrolysis, protein was solubilized and the insoluble non-protein matter was removed, resulting in the high protein content in the resulting hydrolysate (Benjakul and Morrissey, 1997). Fat content in raw material also affected the protein content in resulting hydrolysate. The high amount of lipids resulted in the low amount of solubilized protein (Šližyte *et al*., 2005). Furthermore, pretreatment was also performed to remove phospholipid membrane prior to hydrolysis. As a consequence, both HAP and HFP contained very low amount of fat. Ash content of 1.76-1.78 % in both hydrolysates represented the salt formed during pH adjustment using acid and alkaline. It was noted that both hydrolysates had moisture content of 11.16-11.96%.

Hydrolysates were hygroscopic due to hydrophilic and polar amino acids in the hydrolysate. Therefore, lyophilized hydrolysate could absorb the moisture from the environment easily. Different compositions of fish protein hydrolysate from different sources have been reported such as red salmon (*Oncorhynchus nerka*) head mince (62.3-64.8% protein, 22.5-24.5% fat, 5.0-6.2% moisture and 6.9-7.7% ash) (Sathivel *et al*., 2005), lizardfish (*Saurida elongate*) (84.7% protein, 3.5% fat and 7.1% ash) (Dong *et al*., 2005) and round scad (*Decapterus maruadsi*) muscle (69.0% protein, 0.15% fat, 8.75% moisture and 24.56% ash) (Thiansilakul, *et al*., 2007b).

Table 14. Proximate composition of protein hydrolysate from the muscle of brownstripe red snapper prepared by 2-step hydrolysis process using Alcalase/pyloric caeca protease (PCP) (HAP) and Flavourzyme/PCP (HFP).

Compositions (% wet weight)	HAP	HFP
Protein*	$87.36 \pm 0.12^{a\dagger}$	86.55 ± 0.19^{b}
Moisture*	11.16 ± 0.39^{a}	11.96 ± 0.52^{a}
Fat*	0.61 ± 0.06^{a}	0.64 ± 0.04^{a}
Ash*	1.76 ± 0.73^{a}	1.78 ± 0.32^{a}

Mean ± SD (n=3)
† Different superscripts in the same row indicate the significant differences ($p < 0.05$)

7.4.2 Amino acid composition of protein hydrolysates

Amino acid compositions of HAP and HFP are shown in Table 15. Both HAP and HFP contained glutamic acid/glutamine as the major amino acids and were also rich in aspartic acid/asparagine, alanine, lysine and leucine. Jung *et al*., (2005) reported that glutamic acid and aspartic acid provide the taste of fish and shellfish. However, both hydrolysates contained low levels of cysteine and tryptophan. HAP and HFP constituted 38.01 and 37.87% essential amino acids, respectively. Therefore, they could serve as the excellent source of useful nutrients. The hydrophobic amino acid, including valine, leucine, isoleucine, alanine and

methionine were found at 30.14 and 30.32% of total amino acids in HAP and HFP, respectively. This might contribute to the bitterness of resulting hydrolysates (Saha and Hayashi 2001). Generally, the differences in amino acid composition between HAP and HFP depended on the existing differences in enzyme specificity and hydrolysis conditions (Klompong *et al*., 2009).

Table 15. Amino acid composition of protein hydrolysate from the muscle of brownstripe red snapper prepared by 2-step hydrolysis process using Alcalase/pyloric caeca protease (PCP) (HAP) and Flavourzyme/PCP (HFP)

Amino acids (%)	HAP	HFP
Alanine	9.38	9.56
Arginine	5.00	5.06
Aspartic acid/asparagine	10.65	10.73
Cysteine	0.12	0.14
Glutamic acid/glutamine	16.23	16.92
Glycine	7.48	7.21
Histidine	1.71	1.60
Isoleucine*	4.14	4.07
Leucine*	8.54	8.62
Lysine*	9.51	9.76
Methionine*	2.93	2.92
Phenylalanine*	2.65	2.45
Proline	3.66	3.46
Serine	5.15	5.02
Threonine*	5.10	4.91
Tyrosine	2.33	2.14
Tryptophane	0.28	0.29
Valine*	5.15	5.15

* Essential amino acids

7.4.3 Antioxidative activities of protein hydrolysates

Antioxidative activities of protein hydrolysates from the muscle of brownstripe red snapper, HAP and HFP, are shown in Figure 33. HAP had the higher

DPPH and ABTS radical scavenging activities and FRAP than did HFP ($p < 0.05$), whereas ferrous chelating activity of HFP was higher than that of HAP ($p < 0.05$). Different antioxidative activities between both hydrolysates might be caused by the differences in the exposed side chains of peptides as governed by the specificity of proteases towards protein substrates (Bayram *et al*., 2008). DH also greatly influenced the peptide chain length. The higher DH, indicated the greater cleavage of peptide chains. DH of HAP and HFP were 62.7 and 61.8 %, respectively. Due to the similarity in DH between both hydrolysates, the differences in antioxidative activities were most likely determined by the amino acid sequence and composition in the resulting peptides as well as chain length of individual peptide. Thainsilakul *et al*. (2007a) reported that antioxidative activities were affected by both types of proteases used and the degree of hydrolysis.

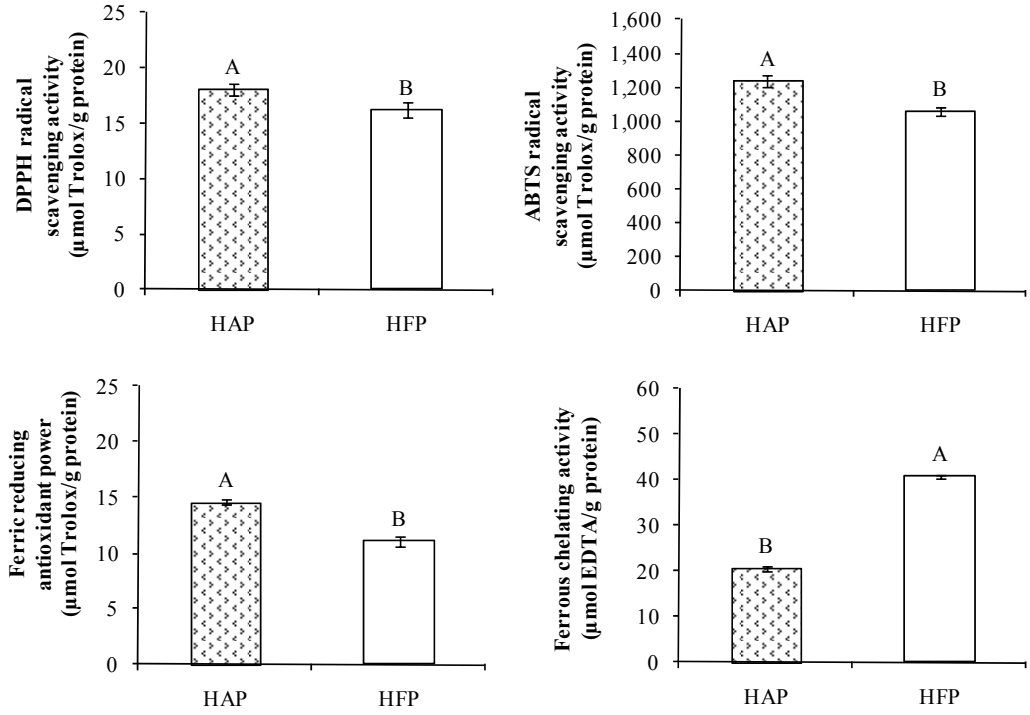

Figure 33. Antioxidative activities of protein hydrolysate from the muscle of brownstripe red snapper prepared by 2-step hydrolysis process using Alcalase/pyloric caeca protease (PCP) (HAP) and Flavourzyme/PCP (HFP). Bars represent the standard deviation (n=3). Different capital letters within the same antioxidative activity assay indicate significant differences ($p < 0.05$).

7.4.4 Antioxidative stability of protein hydrolysate

7.4.4.1 Thermal stability

Antioxidative activities of protein hydrolysate from brownstripe red snapper muscle after incubation at different temperatures for 30 min are depicted in Figure 34. Both HAP and HFP showed no changes in all antioxidative activities tested ($p > 0.05$) at all incubating temperatures. Nevertheless, DPPH radical scavenging activity of HFP was slightly decreased after being incubated at temperature higher than 60°C ($p < 0.05$). This might be due to the alteration of peptides with DPPH radical scavenging activity at high temperature (Binson *et al*., 2008). However, other antioxidative activities were stable over wide range of temperature (25-100°C) ($p < 0.05$). Thus, peptides in HAP and HFP generated with enzymatic hydrolysis were more likely stable to heating process. As a consequence, both HAP and HFP could be used or supplemented as a source of natural antioxidants in thermally processed foods.

HAP and HFP were also subjected to heating at 100°C for different times (60, 90 and 120 min). The remaining antioxidative activities are shown in Figure 35. Antioxidative activities of both HAP and HFP were stable throughout 120 min of heating at 100°C ($p < 0.05$), except for ferrous chelating activity, in which slight decrease was observed when heating time was greater than 60 min ($p < 0.05$). This might result from the changes in peptides with ferrous chelating activity as the heating was conducted for a longer time. Binson *et al*. (2008) reported that accumulated energy from long time heating might induce antioxidative compounds to undergo alteration and loss their activities. Generally, the result suggested that HAP and HFP could function as antioxidants after heating at high temperature for a long time. This result reconfirmed that HAP and HFP could be a promising source of heat stable antioxidative peptides.

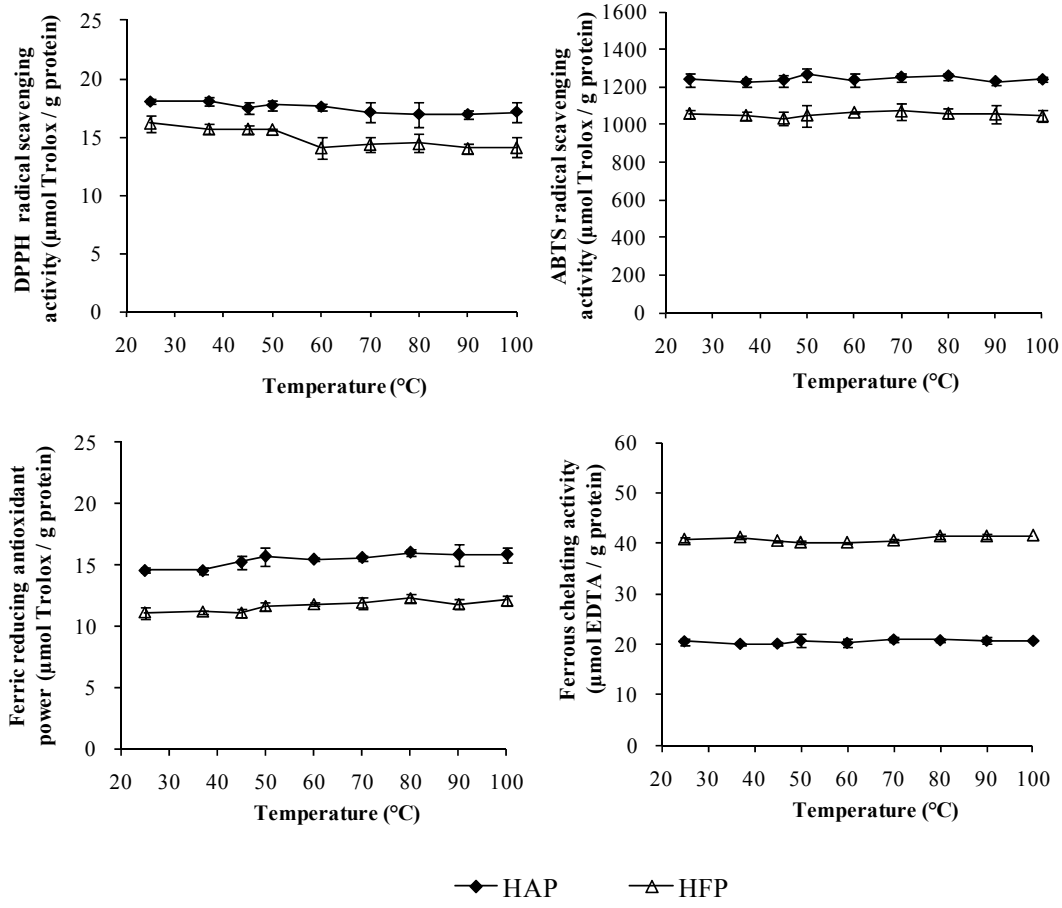

Figure 34. Antioxidative activities of protein hydrolysate from the muscle of brownstripe red snapper prepared by 2-step hydrolysis process using Alcalase/pyloric caeca protease (PCP) (HAP) and Flavourzyme/PCP (HFP) after incubation at different temperatures for 30 min. Bars represent standard deviation (n=3).

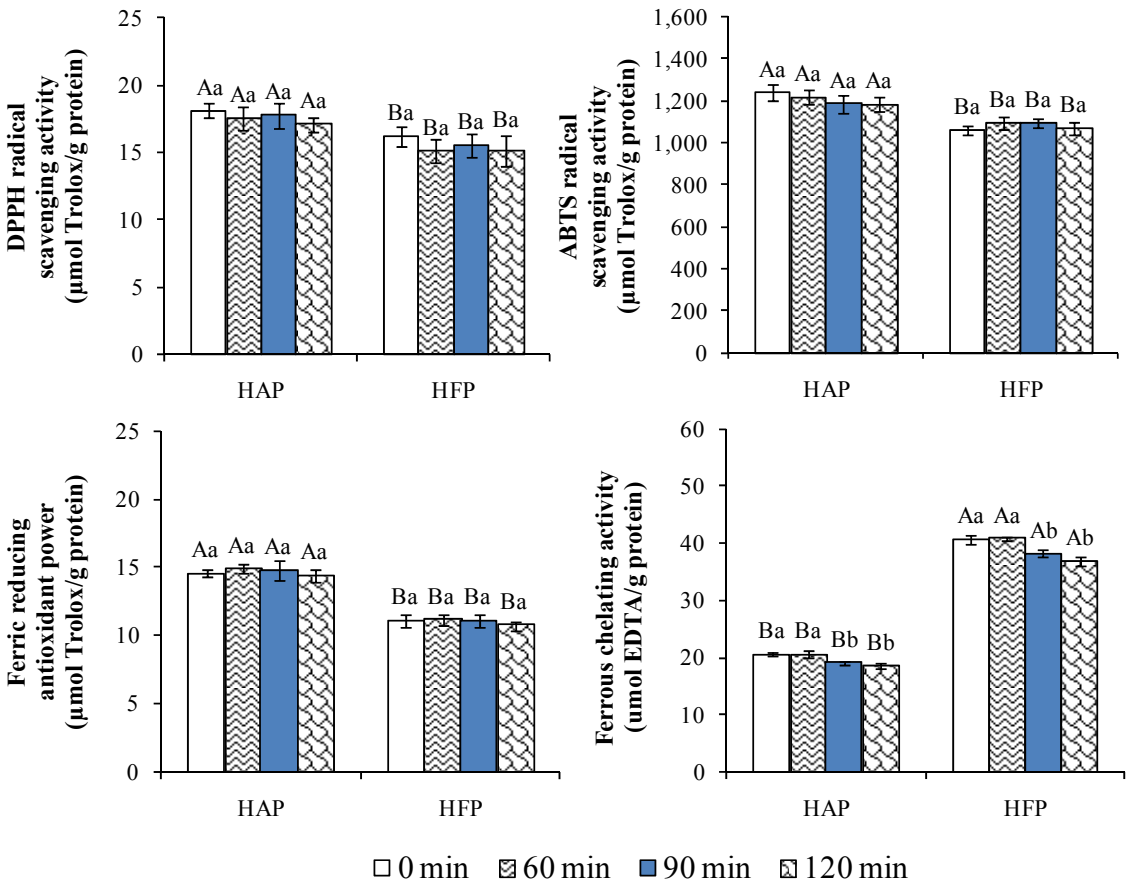

Figure 35. Antioxidative activities of protein hydrolysate from the muscle of brownstripe red snapper prepared by 2-step hydrolysis process using Alcalase/pyloric caeca protease (PCP) (HAP) and Flavourzyme/PCP (HFP) after being heated at 100°C for different times. Bars represent standard deviation (n=3). Different capital letters within the same heating time under the same antioxidative activity assay indicate significant differences ($p < 0.05$). Different letters within the same sample under the same antioxidative activity assay indicate significant differences ($p < 0.05$).

7.4.4.2 pH stability

Antioxidant activities of protein hydrolysate from brownstripe red snapper muscle over a wide range of pH were monitored by measuring DPPH and ABTS radical scavenging activities, FRAP and ferrous chelating activity as shown in Figure 36. DPPH radical scavenging activity and ferrous chelating activity of both HAP and HFP remained constant when subjected to the pH range of 2-12, however a slight increase in chelating activity of HFP in alkaline pH range was observed ($p < 0.05$). For ABTS radical scavenging activity, the alkaline pH resulted in the slight decrease in activity for both hydrolysates ($p < 0.05$). Peptides with the short chains and amino acids in protein hydrolysate are not much affected by charge modification governed by pH changes (Klompong *et al*., 2008). Peptides with ABTS radical scavenging activity and FRAP might undergo the conformation changes at high alkaline and acidic pHs, leading to the loss in such activities. The result suggested that peptides possessing ABTS radical scavenging activity and FRAP in both HAP and HFP might be different from those with DPPH radical scavenging activity and chelating activity in term of configuration, amino acid composition and sequence. In general, antioxidative peptides in HAP and HFP were more likely stable over a wide pH range. Therefore, both HAP and HFP could be used in all foods with different pHs without the dramatic changes in antioxidative activity.

7.4.5 Functional properties of protein hydrolysates

7.4.5.1 Solubility

Solubility is one of the most important functional properties of protein hydrolysates (Kristinsson and Rasco, 2000a). Good solubility of proteins is required in many functional applications, especially for emulsions, foams and gels. Solubility of HAP and HFP at different pHs is shown in Table 16. Both hydrolysates were soluble over a wide pH range with more than 98% solubility. HAP showed no difference in solubility at all pH tested ($p > 0.05$), whereas HFP had the slight decrease in solubility when pH was lower than 4 ($p < 0.05$). The high solubility of

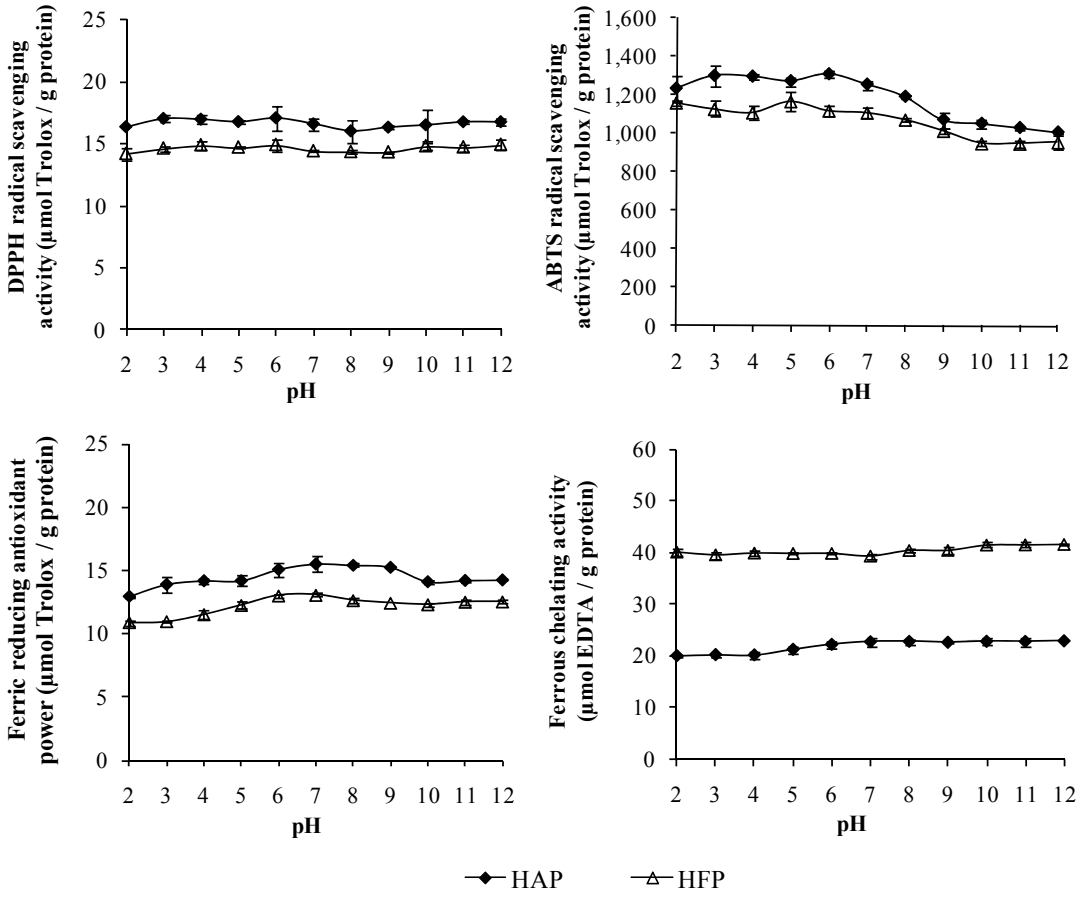

Figure 36. Antioxidative activities of protein hydrolysate from the muscle of brownstripe red snapper prepared by 2-step hydrolysis process using Alcalase/pyloric caeca protease (PCP) (HAP) and Flavourzyme/PCP (HFP) after incubation at different pHs for 30 min. Bars represent standard deviation (n=3).

protein hydrolysate might result from the intense hydrolysis of peptides as indicated by high DH of both HAP and HFP. The cleavage of proteins into smaller peptide usually increases the solubility of fish protein hydrolysates (Shahidi *et al*., 1995). Klompong *et al*. (2007) studied on the solubility of protein hydrolysate prepared from the muscle of yellow stripe travelly and found that hydrolysate with high DH had the higher solubilities than did those possessing the lower DH. Gbogouri *et al*. (2004) reported that hydrolysate from salmon byproduct with higher DH showed higher solubility than that with low DH. Moreover, solubility variations could be attributed

to the net charge of peptides and surface hydrophobicity (Sorgentini and Wagner, 2002). Hydrolysis process directly influences the molecular size, hydrophobicity and polar group of the hydrolysate (Kristensson and Rasco, 2000b). The small peptides from hydrolysate with high DH are expected to have proportionally more polar residues, with the ability to form hydrogen bonds with water, resulted in high solubility (Gbogouri *et al*., 2004; Klompong *et al*., 2007). Additionally, insoluble protein fractions were removed by centrifugation before the protein hydrolysate was lyophilized. Due to the high solubility of the hydrolysate from brownstripe red snapper over a wide pH range, HAP and HFP most likely had the low molecular weight peptides and were hydrophilic in nature.

Table 16. Solubility of protein hydrolysate from the muscle of brownstripe red snapper prepared by 2-step hydrolysis process using Alcalase/pyloric caeca protease (PCP) (HAP) and Flavourzyme/PCP (HFP) at different pHs.

pH	Protein solubility (%)	
	HAP	HFP
2	$98.76 \pm 0.40^{A\dagger a\ddagger}$	98.90 ± 0.35^{Ab}
3	98.73 ± 0.29^{Aa}	98.99 ± 0.33^{Ab}
4	98.94 ± 0.67^{Aa}	98.84 ± 0.24^{Ab}
5	99.42 ± 0.51^{Aa}	99.72 ± 0.26^{Aa}
6	99.44 ± 0.40^{Aa}	99.71 ± 0.23^{Aa}
7	99.61 ± 0.32^{Aa}	99.85 ± 0.14^{Aa}
8	99.50 ± 0.37^{Aa}	99.59 ± 0.22^{Aa}
9	98.91 ± 0.58^{Aa}	99.39 ± 0.44^{Aab}
10	99.13 ± 0.20^{Aa}	99.28 ± 0.24^{Aab}
11	98.62 ± 0.62^{Aa}	99.03 ± 0.70^{Aab}
12	98.49 ± 0.54^{Aa}	98.81 ± 0.67^{Aab}

Means ± SD (n=3)
†Different capital subscripts in the same row indicate the significant differences ($p < 0.05$)
‡Different superscripts in the same column indicate the significant differences ($p < 0.05$)

7.4.5.2 Emulsifying properties

Emulsifying activity index (*EAI*) and emulsion stability index (*ESI*) of both HAP and HFP with different protein concentrations (0.5 and 1%) are presented in Table 17. *EAI* estimates the ability of the protein to aid in the formation and stabilization of newly-created emulsion by giving units of area of the interface that is stabilized per unit weight of protein (Pearce and Kinsella, 1978). *EAI* and *ESI* of both hydrolysates increased with increasing protein concentration ($p < 0.05$). High amount of protein could provide more protein to be adsorbed at the interface (Aewsiri *et al*., 2008). As a result, the thicker films surrounding oil droplet were obtained, leading to emulsion stabilization. However, protein hydrolysate from round scad showed the decrease in *EAI* as protein concentrations increased (Thiansilakul *et al*., 2007b). High solubility of the protein in the dispersing phase also increases the emulsifying efficiency. As a consequence, the protein molecules were able to migrate to the surface of the oil droplets rapidly (Aewsiri *et al*., 2008). At the same protein concentration used, HFP showed the higher *EAI* and *ESI* than did those of HAP, suggesting the difference in intrinsic properties between both protein hydrolysates. The sequence and composition of amino acids in peptide, as affected by protease used, might be different, leading to the difference in emulsifying properties. Klompong *et al*. (2007) indicated that hydrophilic and hydrophobic portions in protein hydrolysate promote oil-in-water emulsion. Additionally, small peptides can migrate and adsorb rapidly at the interface, resulting in high *EAI*. Generally, these small size peptides had less efficiency in stabilizing the interface tension due to lacking of completely reorientation at the interface like large peptides, leading to the low *ESI* (Klompong *et al*., 2007). HFP might form the stronger film with higher uniformity around oil droplets, as evidenced by higher *ESI*. To increase emulsion stability, higher protein concentration of both hydrolysates was recommended.

Table 17. Emulsifying properties of protein hydrolysate from the muscle of brownstripe red snapper prepared by 2-step hydrolysis process using Alcalase/pyloric caeca protease (PCP) (HAP) and Flavourzyme/PCP (HFP) at different protein concentrations.

Protein concentration (%)	*EAI* (m^2/g)		*ESI* (min)	
	HAP	HFP	HAP	HFP
0.5	$19.20 \pm 1.43^{B\dagger b\ddagger}$	24.34 ± 1.11^{Ab}	7.42 ± 1.76^{Ab}	8.61 ± 1.74^{Ab}
1	21.55 ± 1.12^{Ba}	27.61 ± 1.63^{Aa}	14.00 ± 1.15^{Aa}	14.43 ± 0.79^{Aa}

Means ± SD (n=3)
†Different capital subscripts in the same row under the same parameter assayed indicate the significant differences ($p < 0.05$)
‡Different superscripts in the same column indicate the significant differences ($p < 0.05$)

7.4.5.3 Foaming properties

Foam capacity and foam stability of protein hydrolysate from browstripe red snapper at the concentration of 0.5 and 1% are presented in Table 18. Foaming capacities of HAP and HFP with higher concentration were greater than that of lower concentration. An increase in protein concentrations resulted in a higher rate of diffusion to the interface (Sanchez and Patino, 2005). Protein hydrolysate from round scad muscle also showed the higher foam capacity with increasing protein concentration (Thiansilakul *et al*., 2007b). Foaming properties are one of physicochemical characteristics of proteins and hydrolysates, allowing them to form and stabilize foams. Foam formation is governed by three factors, including transportation, penetration and reorganization of molecules at the air-water interface. To exhibit good foaming, proteins have to migrate rapidly to the air-water interface, unfolding and rearranging at the interface (Halling, 1981). Hydrophobic portions of peptides are essential for the adsorption at the air-water interface (Mutiangi *et al*., 1996).

Table 18. Foam properties of protein hydrolysate from the muscle of brownstripe red snapper prepared by 2-step hydrolysis process using Alcalase/pyloric caeca protease (PCP) (HAP) and Flavourzyme/PCP (HFP) at different protein concentrations.

Protein concentration (%)	Foam capacity (%)		Foam stability (%)	
	HAP	HFP	HAP	HFP
0.5	$149.17 \pm 1.44^{B\dagger b\ddagger}$	157.50 ± 2.50^{Ab}	30.53 ± 0.91^{Ab}	32.28 ± 2.65^{Ab}
1	160.83 ± 3.82^{Ba}	182.50 ± 2.50^{Aa}	43.77 ± 2.26^{Aa}	47.82 ± 3.77^{Aa}

Means ± SD (n=3)
†Different capital subscripts in the same row under the same parameter assayed indicate the significant differences ($p < 0.05$)
‡Different superscripts in the same column indicate the significant differences ($p < 0.05$)

Foam stability depends on the protein-protein interaction within the matrix (Mutilangi *et al*., 1996). High molecular weight peptides are generally positively related to foam stability of protein hydrolysates (Klompong *et al*., 2007). Small peptides reduce the foaming stability since they do not have the strength needed to maintain a stable foam (Shahidi *et al*., 1995). Both HAP and HFP showed the greater foam stability when higher concentration of protein was used ($p < 0.05$). The greater stability of foam with increasing protein concentration was due to the denser and more stable interfacial films (Aewsiri *et al*., 2008).

HAP and HFP had some differences in functional properties. Functional properties of protein might be influenced by various factors, including the source of protein, intrinsic properties of protein, the compositions and conformations of protein in solution and at the interface (Sathivel *et al*., 2005; Aewsiri *et al*., 2008). To improve the functional properties of proteins, enzymatic hydrolysis has been extensively employed. Fish protein hydrolysates are generally expected to show higher functional properties, compared to those of native proteins (Thiansilakul *et al*., 2007b).

7.4.6 Changes of protein hydrolysate during extended storage

Changes in antioxidative activities, TBARS and color of both protein hydrolysates, HAP and HFP, were monitored during 12 weeks of storage at 4°C as depicted in Table 19 and 20, respectively. Antioxidantive activities of both HAP and HFP slightly decreased as storage time increased ($p < 0.05$). At week 12, the remaining DPPH and ABTS radical scavenging activities, FRAP and chelating activity of HAP were about 91, 94, 80 and 72%, respectively, compared with initial values (day 0). HFP showed the remaining activities of 87, 94, 84 and 84%, respectively. Loss in antioxidative activities of protein hydrolysate might be due to the aggregation of those hydrolysates. It was noted that the antioxidative activities of both HAP and HFP remained constant up to 8 weeks of storage, except for ferrous chelating activity of HAP that continuously decreased ($p < 0.05$).

TBARS values of both HAP and HFP increased slightly throughout the storage of 12 weeks ($p < 0.05$). At the first day of storage, TBARS values of HAP and HFP were low and no differences in TBARS values between both hydrolysate were observed ($p > 0.05$). The low TBARS more likely resulted from the low fat content of both hydrolysates (0.61-0.64 %). As a consequence, the lower content of polyunsaturated fatty acids was presented in both hydrolysates. This led to the less susceptibility to oxidation as evidenced by lower TBARS values. However, lipid oxidation might take place since HAP and HFP still contained fat at some levels as indicated by the slight increases in TBARS of both hydrolysates.

Some changes in color of HAP and HFP were also observed. At the first day of storage, HFP showed the higher L^* value than did HAP, indicating the greater lightness of HFP than that of HAP. HAP showed the higher a^* and b^* values than did HFP, suggesting the more redness and yellowness of HAP. Generally, HAP was more yellow in color than did HFP. This was due to the dark brown color of Alcalase used for hydrolysis and the longer hydrolysis time conducted. This might lead to the enhanced Maillard reaction between the larger amount of amino groups and carbonyl groups in the mixture. L^* value of silver carp protein hydrolysates was obviously decreased as the hydrolysis time was extended, whereas a^* and b^* values

Table 19. Changes in antioxidative activities, TBARS and color of protein hydrolysate from the muscle of brownstripe red snapper prepared by 2-step hydrolysis process using Alcalase/pyloric caeca protease (PCP) (HAP) during storage of 12 weeks at 4°C

Antioxidative activities / color	Storage time (weeks)				
	0	2	4	8	12
DPPH radical scavenging activity†	19.62 ± 0.22^{a}	19.21 ± 0.19^{ab}	18.79 ± 0.29^{b}	17.85 ± 0.21^{c}	17.88 ± 0.21^{c}
ABTS radical scavenging activity†	1329.36 ± 31.61^{a}	1279.16 ± 22.32^{ab}	1240.71 ± 27.42^{b}	1235.84 ± 26.10^{b}	1242.90 ± 21.72^{b}
Ferric reducing antioxidant power†	15.93 ± 0.40^{a}	15.49 ± 0.37^{a}	14.49 ± 0.43^{b}	13.18 ± 0.52^{c}	12.75 ± 0.45^{c}
Ferrous chelating activity‡	20.43 ± 0.57^{a}	18.05 ± 0.42^{b}	17.40 ± 0.44^{b}	16.20 ± 0.18^{c}	14.68 ± 0.45^{d}
TBARS (mg MDA / kg sample)	1.82 ± 0.19^{c}	2.14 ± 0.07^{b}	2.34 ± 0.09^{a}	2.45 ± 0.09^{a}	2.48 ± 0.14^{a}
*L**	92.76 ± 0.03^{a}	-	90.78 ± 0.03^{b}	88.41 ± 0.02^{c}	88.21 ± 0.02^{d}
*a**	-1.03 ± 0.02^{c}	-	-0.68 ± 0.02^{b}	0.28 ± 0.01^{a}	0.32 ± 0.03^{a}
*b**	13.98 ± 0.08^{d}	-	14.61 ± 0.04^{c}	16.40 ± 0.09^{b}	16.84 ± 0.04^{a}

Means ± SD (n=3)
Different superscripts in the same row indicate significant differences ($p < 0.05$)
†μmol Trolox/g protein
‡μmol EDTA/g protein

Table 20. Changes in antioxidative activities, TBARS and color of protein hydrolysate from the muscle of brownstripe red snapper prepared by 2-step hydrolysis process using Flavourzyme /pyloric caeca protease (PCP) (HFP) during storage of 12 weeks at 4°C

Antioxidative activities / color	Storage time (weeks)				
	0	2	4	8	12
DPPH radical scavenging activity†	16.02 ± 0.18^{a}	15.73 ± 0.11^{a}	14.54 ± 0.12^{b}	14.49 ± 0.27^{b}	13.86 ± 0.38^{b}
ABTS radical scavenging activity†	1021.98 ±4.70^{a}	1002.26 ± 12.63^{b}	965.86 ± 31.99b^{c}	965.92 ± 10.04^{c}	956.16 ± 21.63^{c}
Ferric reducing antioxidant power†	11.47 ± 0.26^{a}	11.14 ± 0.46^{a}	10.41 ± 0.40^{ab}	9.85 ± 0.63^{b}	9.64 ± 0.46^{b}
Ferrous chelating activity‡	47.42 ± 0.47^{a}	46.47 ± 1.38^{ab}	44.13 ± 1.12^{b}	41.00 ± 0.74^{c}	39.97 ± 0.34^{c}
TBARS (mg MDA / kg sample)	1.83 ± 0.22^{b}	2.39 ± 0.17^{a}	2.43 ± 0.08^{a}	2.55 ± 0.19^{a}	2.56 ± 0.10^{a}
*L**	94.67 ± 0.02^{a}	-	90.84 ± 0.04^{b}	88.87 ± 0.01^{c}	88.09 ± 0.04^{d}
*a**	-1.14 ± 0.03^{d}	-	-0.70 ± 0.02^{c}	-0.02 ± 0.03^{b}	0.14 ± 0.023^{a}
*b**	7.24 ± 0.10^{d}	-	13.26 ± 0.03^{c}	14.57 ± 0.02^{b}	15.31 ± 0.02^{a}

Means ± SD (n=3)
Different superscripts in the same row indicate significant differences ($p < 0.05$)
†μmol Trolox/g protein
‡μmol EDTA/g protein

were markedly increased with increasing hydrolysis time (Dong, *et al*., 2008). Additionally, the dark color of some fish protein hydrolysates was possibly from the color of fish itself. Protein hydrolysate from the muscle of round scad, a dark fleshed fish, showed the darker color (L^* = 58.00, a^* = 8.38 and b^* = 28.32) (Thiansilakul *et al*., 2007b). Therefore, the varying color of fish protein hydrolysates depended on the raw material and hydrolysis conditions. During extended storage, color of both hydrolysates became more yellowish as indicated by increases in a^* and b^* values with coincidental decrease in L^* value. The result suggested that Maillard reaction occurred during the extended storage. Lipid oxidation more likely resulted in the formation of lipid oxidation products, especially carbonyl compound, such as aldehydes, etc. Those carbonyls could undergo Maillard reaction with amino group of peptides or free amino acids after hydrolysis via glycation. This resulted in the increased browning of both HAP and HFP during the extended storage.

7.4.7 Fortification of protein hydrolysates with antioxidative activity in soybean milk

7.4.7.1 Physical properties

Physical properties, including color and viscosity of soybean milk fortified with freshly prepared or 8-week stored HAP and HFP at different levels are shown in Table 21 and 22, respectively. Generally, the addition of both freshly prepared and 8-week stored HAP and HFP resulted in the slight decreases of L^* value, whereas b^* values increased slightly with increasing concentration of hydrolysates added ($p < 0.05$), compared with the control sample (without the addition of hydrolysate). However, fortification with freshly prepared and 8-week stored HAP and HFP at different levels had no impact on a^* values of resulting soybean milk ($p < 0.05$). The continuous increase in b^* value as higher amount of hydrolysates was added ($p < 0.05$) indicated the increased yellowness of fortified soybean milk. The increase in yellow color of fortified soybean milk was more likely due to the color of protein hydrolysate added. The fortified soybean milk with 8-week stored HAP and HFP had markedly increased b^* value, compared to that fortified

Table 21. Color and viscosity of soybean milk fortified with different levels of freshly prepared and 8-week stored HAP

Parameters	Control (without hydrolysate)	Freshly prepared			8-week stored		
		0.1%	0.2%	0.3%	0.1%	0.2%	0.3%
*L**	$90.12 \pm 0.01^{a‡}$	$90.03 \pm 0.01^{A†b}$	89.93 ± 0.02^{Ac}	89.73 ± 0.02^{Ad}	90.00 ± 0.06^{Ab}	89.90 ± 0.04^{Ac}	89.70 ± 0.02^{Ad}
*a**	0.92 ± 0.01^{b}	0.99 ± 0.01^{Aa}	1.00 ± 0.02^{Aa}	0.99 ± 0.03^{Aa}	1.01 ± 0.02^{Aa}	1.01 ± 0.02^{Aa}	0.99 ± 0.03^{Aa}
*b**	13.60 ± 0.02^{d}	14.04 ± 0.06^{Bc}	14.19 ± 0.02^{Bb}	14.81 ± 0.02^{Ba}	14.16 ± 0.04^{Ac}	14.26 ± 0.04^{Ab}	15.01 ± 0.03^{Aa}
Viscosity (cP)	682.63 ± 2.50^{c}	687.73 ± 4.76^{Ac}	752.23 ± 2.08^{Ab}	788.37 ± 2.68^{Aa}	689.40 ± 3.50^{Ac}	744.07 ± 16.24^{Ab}	786.83 ± 2.24^{Aa}

Means ± SD (n=3)
†Different capital superscripts within the same level of hydrolysate added in the same row indicate the significant differences ($p < 0.05$)
‡Different superscripts within the same storage time including the control in the same row indicate the significant differences ($p < 0.05$)

Table 22. Color and viscosity of soybean milk fortified with different levels of freshly prepared and 8-week stored HFP

Parameters	Control (without hydrolysate)	Freshly prepared			8-week stored		
		0.3%	0.4%	0.5%	0.3%	0.4%	0.5%
*L**	$90.12 \pm 0.01^{a‡}$	$90.01 \pm 0.01^{A†b}$	89.90 ± 0.00^{Ac}	89.81 ± 0.04^{Ad}	90.01 ± 0.01^{Ab}	89.90 ± 0.01^{Ac}	89.62 ± 0.02^{Bd}
*a**	0.92 ± 0.01^{b}	1.01 ± 0.01^{Aa}	1.02 ± 0.02^{Aa}	1.01 ± 0.02^{Aa}	1.00 ± 0.01^{Aa}	1.02 ± 0.03^{Aa}	1.01 ± 0.02^{Aa}
*b**	13.60 ± 0.02^{d}	13.56 ± 0.06^{Bcd}	14.01 ± 0.03^{Bb}	14.16 ± 0.03^{Ba}	13.97 ± 0.02^{Ac}	14.33 ± 0.11^{Ab}	14.87 ± 0.09^{Aa}
Viscosity (cP)	682.63 ± 2.50^{d}	782.03 ± 2.50^{Ac}	805.10 ± 6.46^{Ab}	821.93 ± 1.42^{Aa}	784.11 ± 1.74^{Ac}	801.67 ± 1.22^{Ab}	820.27 ± 2.65^{Aa}

Means ± SD (n=3)
†Different capital superscripts within the same level of hydrolysate added in the same row indicate the significant differences ($p < 0.05$)
‡Different superscripts within the same storage time including the control in the same row indicate the significant differences ($p < 0.05$)

with freshly prepared counterpart ($p < 0.05$). This was associated with the increase in b^* value of HAP and HFP during the extended storage (Table 19 and 20).

The viscosity of fortified soybean milk with freshly prepared and 8-week stored HAP and HFP at different levels was also determined as shown in Table 16 and 17, respectively. Fortification with protein hydrolysate affected the viscosity of soybean milk to some degrees. Increase in levels of protein hydrolysate added resulted in the higher viscosity of resulting soybean milk ($p < 0.05$). However, no differences were observed between resulting soybean milk fortified with freshly prepared and 8-week stored HAP and HFP at the same concentration ($p > 0.05$).

7.4.7.2 Antioxidative activities

Antioxidative activities of soybean milk fortified with freshly prepared or 8-week stored HAP and HFP at different levels are depicted in Figure 37 and 38, respectively. Fortification of soybean milk with freshly prepared and 8-week stored HAP at all concentrations up to 0.3% had no effects on DPPH and ABTS radical scavenging activities as well as FRAP ($p > 0.05$). However, the higher ferrous chelating activity was observed when the concentration of both freshly prepared and 8-week stored HAP was higher than 0.1% ($p < 0.05$). At the same concentration used, both fresh and stored HAP showed no differences for all assays tested ($p > 0.05$). Although antioxidative activities of stored HAP decreased slightly when storage time increased (Table 19), the remaining antioxidative activities of HAP were high enough to increase ferrous chelating activity of resulting fortified soybean milk.

Soybean milk fortified with freshly prepared and 8-week stored HFP at all levels showed the increase in DPPH radical scavenging activity and ferrous chelating activity ($p < 0.05$), whereas ABTS radical scavenging activity and FRAP remained unchanged at all levels of HFP added ($p < 0.05$). The higher antioxidative activities of soybean milk fortified with both fresh and 8-week stored HFP mainly resulted from the higher amounts of antioxidative protein hydrolysate added. It was noted that soybean milk fortified with stored HFP at the concentration higher than 0.3% showed the lower ferrous chelating activity, compared to that with freshly prepared HFP ($p < 0.05$). Soybean is the potential source of natural antioxidant, e.g.

isoflavone (Prakash *et al*., 2007; Devi *et al*., 2009). Thus, fortification of antioxidative HFP into soybean milk, especially the freshly prepared one, could enhance the antioxidative activities of fortified soybean milk. However, fishy odor/flavor of both HAP and HFP was a main drawback for increasing amount in order to enhance antioxidative activity of resulting product.

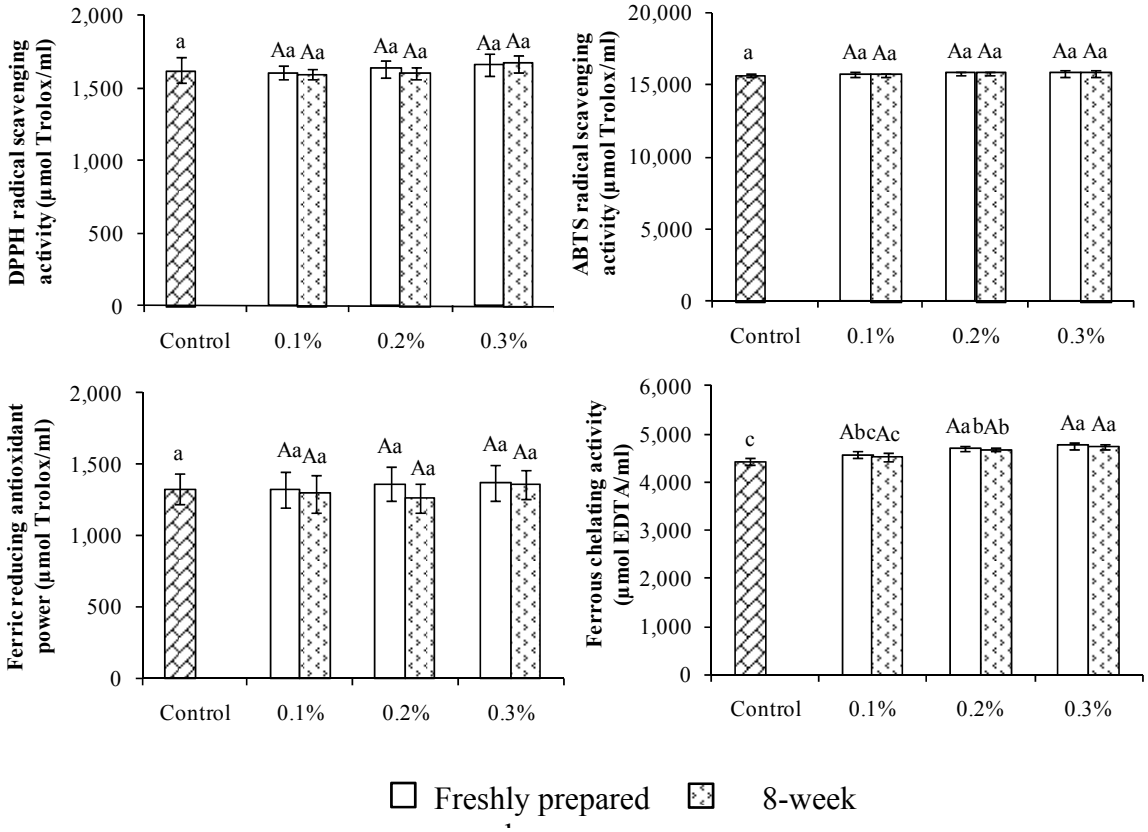

Figure 37. Antioxidative activities of soybean milk fortified with protein hydrolysate from the muscle of brownstripe red snapper prepared by 2-step hydrolysis process using Alcalase/PCP (HAP), freshly prepared and 8-week stored, at different levels. Bars represent the standard deviation (n=3). Different capital letters within the same concentration under the same antioxidative activity assay indicate significant differences ($p < 0.05$). Different letters within the same storage time including the control under the same antioxidative activity assay indicate significant differences ($p < 0.05$).

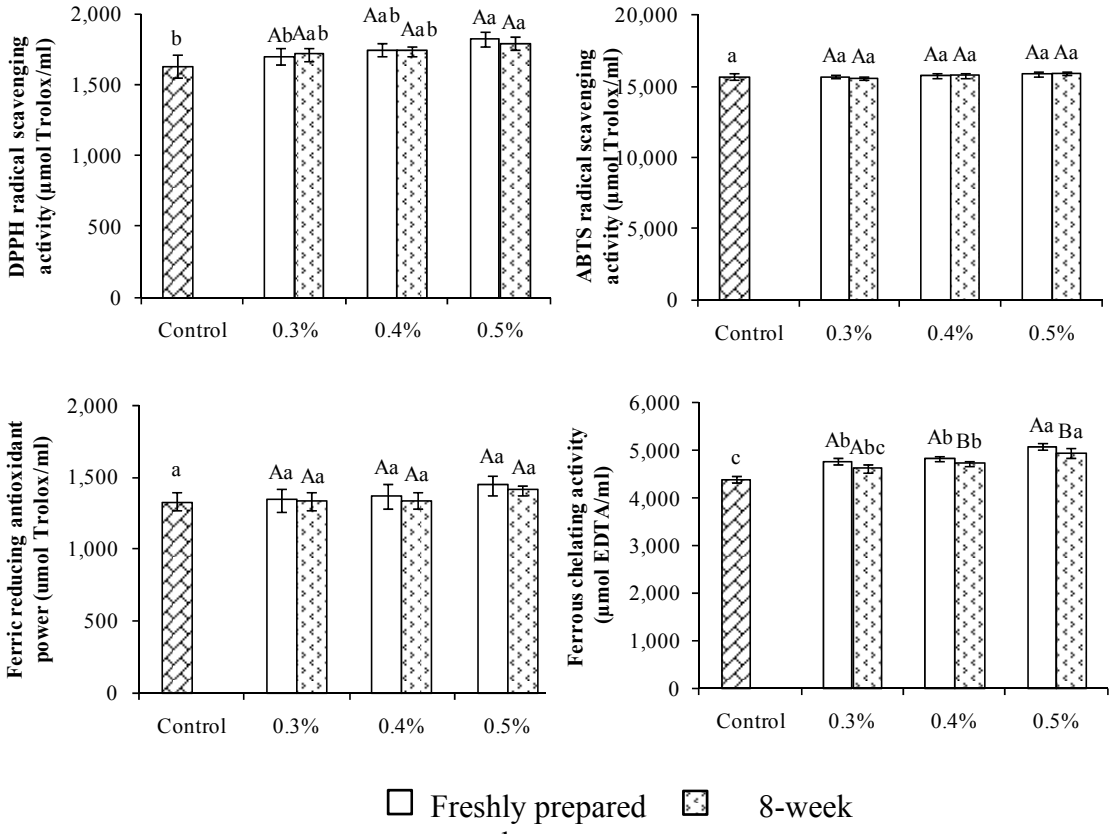

Figure 38. Antioxidative activities of soybean milk fortified with protein hydrolysate from the muscle of brownstripe red snapper prepared by 2-step hydrolysis process using Flavourzyne/PCP (HFP), freshly prepared and 8-week stored, at different levels. Bars represent the standard deviation (n=3). Different capital letters within the same concentration under the same antioxidative activity assay indicate significant differences ($p < 0.05$). Different letters within the same storage time including the control under the same antioxidative activity assay indicate significant differences ($p < 0.05$).

7.4.7.3 Sensory evaluation of fortified soybean milk

Color, odor, flavor, taste and overall likeness of the control soybean milk and soybean milk fortified with freshly prepared or 8-week stored HAP and HFP at different levels (0.1, 0.2 and 0.3% for HAP and 0.3, 0.4 and 0.5% for HFP) are shown in Table 23 and 24, respectively. The selected concentrations of protein

hydrolysate were considered from the maximal level of hydrolysate added in soybean milk, in which fishy odor and bitterness could not be detected by consumers (data not shown). Those levels were 0.3% for HAP and 0.5% for HFP. The results indicated that the fortification of freshly prepared HAP up to 0.3% had no impact on likeness of all attributes of fortified soybean milk ($p > 0.05$). However, 8-week stored HAP at a level of 0.3% lowered the flavor score of soybean milk ($p < 0.05$) but showed no impact on overall likeness ($p < 0.05$). The fishy odor of stored HAP might contribute to the decreased flavor likeness. Fortification of freshly prepared HFP greater than 0.4% affected the flavor and taste likeness of soybean milk, whereas the addition of 8-week stored HFP at levels greater than 0.3% decreased the taste score of product ($p < 0.05$). However, soybean milk fortified with freshly prepared and stored HFP at the level of 0.4% showed no differences in overall likeness score compared with the control ($p > 0.05$).

The higher level of protein hydrolysate fortified, the stronger fishy odor and bitterness were obtained. This problem limits the use of protein hydrolysate, especially product with higher DH. The bitterness in protein hydrolysates was attributed to the hydrophobic amino acids of peptides (Saha and Hayashi, 2001). Hydrolysis process might induce the exposure of the buried hydrophobic peptides, resulting in detection of bitter taste by human taste buds. The strong smell (fishy odor) and bitterness in resulting hydrolysate were the major problems to overcome. With protein hydrolysate having the lowered fishy odor and bitterness, a higher amount of hydrolysate could be incorporated in foods, especially for several kinds of drinks with neutraceutical property. From the results, protein hydrolysate from the muscle of brownstripe red snapper could be used in soybean milk to enhance antioxidative activities at a level of 0.3% for HAP and 0.4% for HFP without the detrimental effect on sensory property. However, freshly prepared hydrolysates were recommended to be used for the fortification.

Table 23. Color, odor, flavor, taste and overall likeness of soybean milk fortified with different levels of freshly prepared and 8-week stored HAP.

Attributes	Control (without hydrolysate)[A]	Freshly prepared			8-week stored		
		0.1%	0.2%	0.3%	0.1%	0.2%	0.3%
Color	$8.15 \pm 0.88^{a‡}$	$8.20 \pm 0.79^{A†a}$	8.20 ± 0.96^{Aa}	8.25 ± 0.59^{Aa}	8.15 ± 0.81^{Aa}	8.10 ± 0.64^{Aa}	7.95 ± 1.43^{Aa}
Odor	8.15 ± 0.79^{a}	8.25 ± 0.69^{Aa}	8.05 ± 1.02^{Aa}	7.65 ± 0.59^{Aa}	7.85 ± 0.81^{Aa}	7.20 ± 1.10^{Aa}	6.45 ± 1.05^{Aa}
Flavor	8.20 ± 0.85^{a}	7.85 ± 0.80^{Aa}	7.85 ± 0.97^{Aa}	7.40 ± 0.59^{Aa}	7.80 ± 0.95^{Aa}	7.05 ± 0.85^{Aab}	5.85 ± 0.81^{Bb}
Taste	8.15 ± 0.81^{a}	8.05 ± 1.15^{Aa}	7.65 ± 0.78^{Aa}	7.45 ± 1.02^{Aa}	7.75 ± 0.91^{Aa}	7.05 ± 1.23^{Aa}	6.65 ± 1.03^{Aa}
Overall	8.25 ± 0.88^{a}	8.00 ± 1.13^{Aa}	7.85 ± 0.96^{Aa}	7.65 ± 1.02^{Aa}	7.85 ± 0.88^{Aa}	7.05 ± 1.23^{Aa}	6.85 ± 1.03^{Aa}

Means ± SD (n=30)
[A]Scores were based on a 9-point hedonic scale (1: Dislike extremely, 5: Neither like nor dislike, 9: Like extremely)
†Different capital superscripts within the same level of hydrolysate added in the same row indicate the significant differences ($p < 0.05$)
‡Different superscripts within the same storage time including the control in the same row indicate the significant differences ($p < 0.05$)

Table 24. Color, odor, flavor, taste and overall likeness of soybean milk fortified with different levels of freshly prepared and 8 weeks stored HFP.

Attributes	Control (without hydrolysate)[A]	Freshly prepared			8-week stored		
		0.3%	0.4%	0.5%	0.3%	0.4%	0.5%
Color	$8.25 \pm 0.79^{a‡}$	$8.20 \pm 1.01^{A†a}$	8.25 ± 0.85^{Aa}	7.95 ± 1.43^{Aa}	8.25 ± 0.92^{Aa}	8.30 ± 0.80^{Aa}	8.45 ± 0.60^{Aa}
Odor	8.10 ± 0.85^{a}	8.00 ± 1.12^{Aa}	6.45 ± 2.11^{Aa}	6.45 ± 1.31^{Aa}	7.95 ± 1.23^{Aa}	7.30 ± 1.42^{Aa}	5.85 ± 1.84^{Aa}
Flavor	8.05 ± 0.60^{a}	7.75 ± 0.97^{Aab}	6.25 ± 1.84^{Aab}	5.80 ± 1.07^{Ab}	7.65 ± 0.75^{Aa}	6.05 ± 1.43^{Aab}	5.05 ± 1.54^{Ab}
Taste	8.05 ± 0.69^{a}	7.60 ± 0.88^{Aa}	6.00 ± 1.12^{Aab}	5.90 ± 1.13^{Ab}	7.45 ± 0.89^{Aab}	6.20 ± 1.15^{Abc}	4.85 ± 1.27^{Ac}
Overall	8.30 ± 0.80^{a}	7.90 ± 1.13^{Aab}	6.60 ± 1.79^{Aab}	5.85 ± 1.07^{Ab}	7.70 ± 0.92^{Aab}	6.55 ± 1.19^{Aab}	5.50 ± 1.28^{Ab}

Means ± SD (n=30)
[A]Scores were based on a 9-point hedonic scale (1: Dislike extremely, 5: Neither like nor dislike, 9: Like extremely)
†Different capital superscripts within the same level of hydrolysate added in the same row indicate the significant differences ($p < 0.05$)
‡Different superscripts within the same storage time including the control in the same row indicate the significant differences ($p < 0.05$)

7.5 Conclusion

Protein hydrolysate from the muscle of brownstripe red snapper prepared by two-step hydrolysis process using Alcalase prior to pyloric caeca protease (PCP) (HAP) and using Flavourzyme prior to PCP (HFP) possessed antioxidative activities. The activities were more likely stable during 3 months of storage, while slight browning took place during storage. Both HAP and HFP showed the great solubility over wide pH ranges and possessed emulsifying and foaming properties. Fortification in soybean milk could enhance the antioxidative activities to some degrees. However, the bitterness and fishy odor of resulting product still limited the levels of hydrolysate fortified.

CHAPTER 8

SUMMARY AND FUTURE WORKS

8.1 Summary

1. Pyloric caeca extracts from brownstripe red snapper, bigeye snapper and threadfin bream were the source of trypsin or trypsin-like proteases. Pyloric caeca proteases could hydrolyze skin gelatin to some extent and the resulting hydrolysate exhibited antioxidative activity, which varied depending upon both sources of proteases and gelatins. Nevertheless, the lower antioxidative activity was observed in gelatin hydrolysate produced, in comparison with protein hydrolysate from other sources.
2. The proteases from pyloric caeca of brownstripe red snapper were purified and classified to be trypsin. Purified trypsin exhibited the highest hydrolytic activity toward BAPNA at 60°C and pH 8.5. K_m and k_{cat} were 0.507 mM and 4.71 s^{-1}, respectively, when BAPNA was used as the substrate and were 0.328 mM and 112 s^{-1}, respectively, when TAME was used.
3. To lower the pro-oxidant and membrane lipid of brownstripe red snapper mince prior to hydrolysis, mince was subjected to membrane separation with subsequent washing. This pretreated mince could be used as the promising proteinaceous substrate for production of hydrolysate with negligible pro-oxidants.
4. Protein hydrolysates from the pretreated brownstripe red snapper mince prepared by two-step hydrolysis process involving commercial protease and pyloric caeca protease (PCP) of brownstripe red snapper provided the peptides with antioxidative and ACE inhibitory activities. After digestion in gastrointestinal tract model system, the hydrolysates exhibited the stronger antioxidative activities. Thus, bioactivities of hydrolysate was maintained or enhanced after ingestion.

5. Protein hydrolysate from the pretreated mince of brownstripe red snapper prepared by two-step hydrolysis process using Alcalase/PCP or Flavourzyme/PCP contained antioxidative peptides. Radical scavenging peptides from Alcalase/PCP hydrolysate included Asn-Arg-Lys-Arg, Asp-Ala-Gly-Leu-Phe-Lys, Met-Glu-Glu-Asp-Leu-Glu-Glu-Arg and Met-Ser-Leu-Trp-Gln-Ser-Leu-Met-Asn-Asp-Lys. Ferrous chelating peptides from Flavourzyme/PCP hydrolysate included Cys-Gly-Asp-Ser-Val-Lys, Met-Cys-Cys-Cys-Arg, His-Arg-Arg-Arg, Asn-Phe-Cys-Ser-Arg, Trp-Trp-Arg-Lys and Phe-Cys-Gly-Val-Ala-Thr-Lys.
6. Protein hydrolysate from the pretreated brownstripe red snapper mince prepared by two-step hydrolysis process showed the great solubility over wide pH ranges and possessed emulsifying and foaming properties. Antioxidative activities of hydrolysate were more likely stable during 3 months of storage at 4°C. The fortification in soybean milk increased the antioxidative activity of the drink to some degrees, while the consumer acceptability was still achieved.

8.2 Future works

1. Trypsin of other fish species from surimi processing by-products should be studied.
2. Pretreatment of mince from other fish species, especially dark flesh fish, prior to hydrolysis process should be conducted to enhance antioxidative activity of resulting protein hydrolysate by lowering pro-oxidant and lipids susceptible to oxidation.
3. Several steps of hydrolysis using other proteases in conjunction with fish proteases should be performed to provide protein hydrolysate with desirable antioxidative activities.
4. The bitterness and fishy odor of resulting protein hydrolysate should be reduced to expand its applications, especially in food systems.
5. Identified antioxidative peptides should be synthesized and evaluated for their antioxidative activities.

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VITAE

Name Ms. Sutheera Khantaphant

Student ID 4911030008

Educational Attainment

Degree	Name of Institution	Year of Graduation
Bachelor of Science (Food Science and Technology)	Chiangmai University	2003

Scholarship during Enrolment

CHE Ph.D. Scholarship by Office of the Higher Education Commission, Ministry of Education, Thailand.

List of Publication and Proceedings

Publications

1. Khantaphant, S. and Benjakul, S. 2008. Comparative study on the proteases from fish pyloric caeca and the use for production of gelatin hydrolysate with antioxidative activity. Comp. Biochem. Physiol. B. 151: 410-419.
2. Khantaphant, S. and Benjakul, S. 2010. Purification and characterization of trypsin from the pyloric caeca of brownstripe red snapper (*Lutjanus vitta*). Food Chem. 120: 658-664.
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Proceedings

1. Khantaphant, S. and Benjakul, S. 2008. Characterization of proteases from pyloric caeca of bigeye snapper (*Priacanthus tayenus*) and threadfin bream (*Nemipterus marginatus*). The Sixth Regional IMT-GT Uninet Conference 2008, Universiti Sains Malaysia, Penang, Malaysia: 28th-30th August, 2008. Oral presentation.
2. Khantaphant, S. and Benjakul, S. 2009. Antioxidative activity of protein hydrolysate from brownstripe red snapper (*Lutjanus vitta*) as affected by prior washing and defatting. International Symposium on Seafood Processing Technology and Safety Control System, Ocean University of China, Qingdao, China: 31st October - 2nd November, 2009. Oral presentation.