Purification, Partitioning and Uses of Fish Pepsin for Collagen Extraction and Protein Hydrolysate Production

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ชื่อวิทยานิพนธ์	การทำบริสุทธิ์ การแยกส่วน และการใช้เปปซินจากปลาเพื่อการสกัดคอลลาเจนและการผลิตโปรตีนไฮโดรไลเสต
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บทคัดย่อ

จากการศึกษาคุณลักษณะของเอนไซม์โปรตีเนสจากส่วนสกัดกระเพาะปลาทูน่าพันธุ์ครีบยาว (*Thunnus alalunga*) ปลาทูน่าพันธุ์โอแถบ (*Katsuwonus pelamis*) และปลาทูน่าพันธุ์โอดำ (*Thunnus tonggol*) ที่ พีเอช 2.0 และอุณหภูมิ 50 °ซ โดยใช้ฮีโมโกลบินเป็นสับสเตรท พบว่าส่วนสกัดกระเพาะปลาทูน่าพันธุ์ครีบยาวมีกิจกรรมของเอนไซม์โปรตีเนสสูงสุด โดยส่วนสกัดกระเพาะปลาทูน่าพันธุ์โอดำ และโอแถบมีกิจกรรมของเอนไซม์โปรตีเนสรองลงมาตามลำดับ จากการศึกษาผลของสารยับยั้งต่อกิจกรรมของเอนไซม์โปรตีเนสจากส่วนสกัดกระเพาะปลาทูน่าทั้งสามสายพันธุ์ พบว่าโปรตีเนสชนิดแอสปาร์ติกที่มีลักษณะคล้ายเปปซินเป็นเอนไซม์หลัก โปรติเนสเหล่านี้ถูกยับยั้งได้โดยโซเดียมโดเดซิลซัลเฟตและซีสเตอีน เมื่อทำบริสุทธิ์เปปซิโนเจนจากปลาทูน่าพันธุ์ครีบยาว และเปปซินสองชนิดจากปลาทูน่าพันธุ์โอแถบ โดยการใช้เทคนิคโครมาโทกราฟีต่างๆ จนกระทั่งได้เอนไซม์ที่ผ่านการทำบริสุทธิ์ปรากฏเป็นแถบโปรตีนเดี่ยวบนเจล native-PAGE และ SDS-PAGE เมื่อตรวจสอบโดย SDS-PAGE พบว่าเปปซิโนเจนและแอคทีฟเปปซินจากปลาทูน่าพันธุ์ครีบยาวมีน้ำหนักโมเลกุลเท่ากับ 39.9 และ 32.7 กิโลดาลตัน ตามลำดับ เปปซิโนเจนเปลี่ยนไปเป็นเปปซินโดยผ่านรูปตัวกลาง (น้ำหนักโมเลกุลประมาณ 36.8 กิโลดาลตัน) และการเปลี่ยนเป็นเปปซินเกิดขึ้นอย่างสมบูรณ์ภายหลัง 30-60 นาที เปปซิน 1 และ 2 จากปลาทูน่าพันธุ์โอแถบมีน้ำหนักโมเลกุลเท่ากับ 33.9 และ 33.7 กิโลดาลตัน ตามลำดับ เปปซิโนเจนจากปลาทูน่าพันธุ์ครีบยาวมีลำดับกรดอะมิโนปลายสายด้านหมู่อะมิโนจำนวน 15 หน่วยย่อยแรกของส่วนการกระตุ้นคือ FHKLPLIKGKTAREE ส่วนลำดับกรดอะมิโน 20 ตัวแรกของทั้งเปปซิน 1 และ 2 จากปลาทูน่าพันธุ์โอแถบคือYQDGTEPMTNDADLSYYGVI เปปซินทั้งหมดมีพีเอชและอุณหภูมิที่เหมาะสมในช่วง 2.0-2.5 และ 45-50 °ซ ตามลำดับ และมีความคงตัวต่อพีเอชในช่วง 2-5 และอุณหภูมิสูงถึง 50 °ซ เปปสเตติน เอ สามารถยับยั้งกิจจกรรมของเปปซินบริสุทธิ์โดยขึ้นกับระดับความเข้มข้น เปปซิน 1 และ 2 จากปลาทูน่าพันธุ์โอแถบมีความจำเพาะสูงต่อการย่อยสลายฮีโมโกลบิน โดยมีค่า K_m เท่ากับ 54 และ 71 ไมโครโมลาร์ ตามลำดับ และมีค่า k_{cat} เท่ากับ 38.1 และ 44.3 ต่อวินาที

ตามลำดับ เปปซินทั้งสองสามารถย่อยสลายโปรตีนซีรัมอัลบูมินจากวัว ไข่ขาว แอคโตไมโอซินธรรมชาติจากปลากะพงข้างเหลือง คอลลาเจนชนิดละลายด้วยกรดจากปลากรีนลิ่ง อย่างไรก็ตามระดับการย่อยสลายยังคงต่ำกว่าเปปซินจากกระเพาะหมู

จากการศึกษาการแยกส่วนเปปซินจากกระเพาะปลาทูน่าพันธุ์ครีบยาวโดยการใช้ระบบของเหลวสองเฟส (ATPS) พบว่า ATPS ที่ดีที่สุดสำหรับการแยกส่วนเปปซินจากสารสกัดกระเพาะ (SE) และสารสกัดกระเพาะที่ผ่านการปรับสภาวะกรด (ASE) ประกอบด้วย PEG1000 ร้อยละ 25 $MgSO_4$ ร้อยละ 20 และ PEG2000 ร้อยละ 15 $MgSO_4$ ร้อยละ 15 ซึ่งสามารถเพิ่มระดับความบริสุทธิ์ได้ 7.2 และ 2.4 เท่า โดยได้ผลผลิตร้อยละ 85.7 และ 89.1 ตามลำดับ จากการศึกษาเทคนิคอิเล็กโตรโฟรีซีสพบว่า SE มีโปรตีนหลักที่มีน้ำหนักโมเลกุลเท่ากับ 40.6 กิโลดาลตัน ขณะที่โปรตีนที่มีน้ำหนักโมเลกุลเท่ากับ 32.7 กิโลดาลตัน เป็นโปรตีนหลักใน ASE การประยุกต์ใช้ ATPS มีผลให้เปปซิโนเจนใน SE ถูกกระตุ้นโดยกระบวนการปรับกรดและการแยกส่วน จากการศึกษาความคงตัวพบว่า SE มีความคงตัวที่อุณหภูมิ 0 °ซ และ 4 °ซ เป็นเวลา 14 วัน การสูญเสียกิจกรรมของเอนไซม์โปรติเอสใน ASE และส่วนที่แยกได้จาก ATPS เพิ่มขึ้นเมื่อระยะเวลาและอุณหภูมิการเก็บรักษาเพิ่มขึ้น ดังนั้น ATPS สามารถใช้ในการแยกส่วนและทำบริสุทธิ์เบื้องต้นเปปซินจากกระเพาะปลาทูน่าพันธุ์ครีบยาวได้อย่างมีประสิทธิภาพ

การสกัดคอลลาเจนจากหนังปลาทรายแดง (*Nemipterus spp.*) โดยใช้เปปซินจากปลาทูน่า (10 ยูนิตต่อกรัมหนังที่ผ่านการกำจัดไขมัน) เป็นเวลา 12 ชม. ให้ผลผลิตเพิ่มขึ้น 1.84-2.32 เท่า โดยเปปซินจากปลาทูน่าพันธุ์ครีบยาวมีประสิทธิภาพเทียบเท่าเปปซินจากหมู โดยทั่วไปผลผลิตเพิ่มขึ้นเมื่อระยะเวลาในการสกัดเพิ่มขึ้น ($P < 0.05$) การสกัดคอลลาเจนจากหนังปลาวัวหนัง (*Aluterus monoceros*) โดยการใช้เปปซินจากปลาทูน่าพันธุ์ครีบยาวที่ระดับ 20 ยูนิตต่อกรัมหนัง ให้ผลผลิตร้อยละ 28.8 (โดย นน. แห้ง) ซึ่งสูงกว่าวิธีการสกัดด้วยกรดประมาณ 8 เท่า ผลผลิตของคอลลาเจนชนิดละลายด้วยกรด (ASC) และคอลลเจนชนิดละลายด้วยเปปซิน (PSC) ของปลาทูน่าพันธุ์ครีบยาว ที่สกัดจากหนังปลากรีนลิ่ง (*Pleurogrammus azonus*) เท่ากับร้อยละ 30.3 และ 14.0 (โดย นน. แห้ง) ตามลำดับ จากการศึกษารูปแบบโปรตีนและการใช้คอร์ลัมน์โครมาโทกราฟี TOYOPEARL® CM-650M พบว่าคอลลาเจนทั้งสองชนิดประกอบด้วยสายแอลฟาและบีต้าเป็นองค์ประกอบหลักและสามารถจำแนกได้เป็นชนิด I เมื่อตรวจสอบด้วยเทคนิคฟูเรียร์ทรานฟอร์มอินฟราเรด (FTIR) สเปกโตรสโกปีพบว่า ASC และ PSC จากปลาทั้งสองสายพันธุ์ประกอบด้วยเกลียวสามสายเป็นองค์ประกอบหลัก แต่พบความแตกต่างเล็กน้อยในโครงสร้างระดับทุติยะภูมิระหว่างคอลลาเจนทั้งสองชนิด จากการวิเคราะห์ศักย์ซีต้าพบว่าค่า p*I* ของคอลลาเจนจากปลาทั้งสองสายพันธุ์คาดคะเนอยู่ในช่วงพีเอช 6.29 ถึง 6.38

จากการศึกษาการย่อยสลายคอลลาเจนชนิด I ด้วยเปปซินชนิดต่างๆ คือเปปซินจากหมู เปปซิน 1 และ 2 จากปลาทูน่าพันธุ์โอแถบ พบว่าคอลลาเจนบริสุทธิ์ชนิด I จากหนังปลาทรายแดงโม่ง (*N. hexodon*) มีโครงสร้างหลักคือ $[\alpha 1(I)]_2\alpha 2(I)$ โดยมี $\alpha 1(I)\alpha 2(I)\alpha 3(I)$ เป็นองค์ประกอบร่วม ภายหลังจากการย่อยสลายคอลลาเจนบริสุทธิ์ชนิด I พบว่า สัดส่วนของสาย $\alpha 1/\alpha 2$ มีความเข้มแถบเพิ่มสูงขึ้น ($P < 0.05$) พร้อมกับการลดลงของความเข้มแถบขององค์ประกอบเชื่อมข้ามที่มีน้ำหนักโมเลกุลสูงกว่า องค์ประกอบเชื่อมข้ามถูกย่อยสลายอย่างมีประสิทธิภาพด้วยเปปซิน 1 และ 2 จากปลาทูน่าพันธุ์โอแถบ และเปปซินจากหมูที่อุณหภูมิ 4 °ซ โดยไม่มีการตัดสายบีต้าและแอลฟา อย่างไรก็ตามที่อุณหภูมิ 50 °ซ คอลลาเจนบริสุทธิ์ชนิด I สามารถถูกย่อยสลายได้อย่างง่ายด้วยเปปซินจากหมู เปปซิน 2 และ 1 จากปลาทูน่าพันธุ์โอแถบตามลำดับ

จากการศึกษาสมบัติเชิงหน้าที่ และกิจกรรมการต้านอนุมูลอิสระของโปรตีนไฮโดรไลเสตจากกล้ามเนื้อปลาปลาทรายแดงโม่ง ที่ผ่านการเตรียมโดยใช้เปปซินจากปลาทูน่าพันธุ์โอแถบที่ระดับการย่อยสลายต่างๆ (ร้อยละ 10 20 และ 30) พบว่าการละลายของไฮโดรไลเสตผันแปรจากร้อยละ 71 ถึง 99 ในช่วงพีเอช 3-9 โดยขึ้นอยู่กับระดับการย่อยสลาย คุณสมบัติการเกิดอิมัลชันและการเกิดโฟมของไฮโดรไลเสตขึ้นกับระดับการย่อยสลาย และความเข้มข้นที่ใช้ ไฮโดรไลเสตที่มีระดับการย่อยสลาย ร้อยละ 20 มีค่ากิจกรรมการจับอนุมูลอิสระ ABTS และ DPPH สูงสุด อย่างไรก็ตามกิจกรรมการจับ Fe^{2+} ของไฮโดรไลเสตเพิ่มขึ้นเมื่อระดับการย่อยสลายเพิ่มขึ้น ($P < 0.05$) เมื่อนำไฮโดรไลเสตที่มีระดับการย่อยสลาย ร้อยละ 20 มาแยกด้วยโครมาโตกราฟีแบบจำกัดขนาดโดยใช้ Sephadex G-25 พบว่าเปปไทด์ที่มีกิจกรรมการต้านอนุมูลอิสระที่มีน้ำหนักโมเลกุลประมาณ 1.3 กิโลดาลตัน แสดงค่ากิจกรรมการจับอนุมูลอิสระ ABTS สูงสุด แฟรคชั่นที่มีเปปไทด์ที่มีกิจกรรมการต้านอนุมูลอิสระมีความคงตัวระหว่างพีเอชช่วงกว้าง (1-10) และมีความคงตัวต่อความร้อนที่อุณหภูมิ 100 °ซ เป็นเวลา 3 ชม. การศึกษาการจำลองการย่อยสลายในกระเพาะอาหารและลำไส้พบว่าการย่อยสลายโดยเปปซินไม่มีผลต่อกิจกรรมการจับอนุมูลอิสระ ABTS ของเปปไทด์ที่มีกิจกรรมการต้านอนุมูลอิสระ ขณะที่การย่อยสลายต่อเนื่องด้วยแพนครีเอตินส่งผลเพิ่มกิจกรรมการจับอนุมูลอิสระ ดังนั้นโปรตีนไฮโดรไลเสตที่ผลิตโดยเปปซินจากปลาทูน่าพันธุ์โอแถบสามารถใช้เป็นแหล่งของเปปไทด์ที่มีกิจกรรมการต้านอนุมูลอิสระที่มีศักยะภาพ

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ABSTRACT

Proteinases from the stomach extract of albacore tuna (*Thunnus alalunga*), skipjack tuna (*Katsuwonus pelamis*) and tongol tuna (*Thunnus tonggol*) were studied. Based on activity assayed at pH 2.0 and 50 °C using hemoglobin as a substrate, albacore tuna stomach extract showed the highest activity, followed by those of tongol tuna and skipjack tuna, respectively. Inhibitor study revealed that major proteinases in the stomach of all tuna species were aspartic proteinases, most likely pepsin. These proteinases were strongly inhibited by SDS and cysteine. Pepsinogen (PG) from albacore tuna and two pepsins from skipjack tuna stomach were purified to homogeneity by using a series of chromatographies to obtain a single band on native-polyacrylamide gel electrophoresis (native-PAGE) and sodium dodecyl sulfate (SDS)-PAGE. Molecular weights (MW) of PG and active pepsin from albacore tuna were estimated to be 39.9 and 32.7 kDa as determined by SDS-PAGE, respectively. PG was converted to the corresponding pepsin through an intermediate form (MW≈36.8 kDa) and the complete activation was observed after 30-60 min. Pepsins 1 and 2 from skipjack tuna were estimated to be 33.9 and 33.7 kDa, respectively. The N-terminal amino acid sequence of the first 15 amino acids of activation segment of PG from albacore tuna was FHKLPLIKGKTAREE, whereas the first 20 amino acids of both pepsin 1 and 2 from skipjack tuna were YQDGTEPMTNDADLSYYGVI. The optimal pH and temperature for all pepsins were in range of 2.0-2.5 and 45-50 °C, respectively, and they were stable in the pH range of 2-5 and at temperatures up to 50 °C. The activity of all purified pepsins was strongly inhibited by pepstatin A in a dose-dependent manner. Pepsins 1 and 2 from skipjack tuna had high affinity and hydrolytic activity toward hemoglobin with K_m of

54 and 71 μM, respectively. k_{cat} of pepsins 1 and 2 were 38.1 and 44.3 s^{-1}, respectively. Both pepsins effectively hydrolyzed bovine serum albumin, egg white, natural actomyosin from brownstripe red snapper muscle and acid-solubilized collagen from arabesque greenling skin. Nevertheless, the hydrolytic activity was slightly less than that of pepsin from porcine stomach.

Partitioning of pepsin from stomach of albacore tuna using an aqueous two-phase system (ATPS) was investigated. The best ATPS conditions for pepsin partitioning from stomach extract (SE) and acidified counterpart (ASE) were 25% PEG1000-20% $MgSO_4$ and 15% PEG2000-15% $MgSO_4$, which increased the purity by 7.2-fold and 2.4-fold with the recovered activity of 85.7% and 89.1%, respectively. Electrophoretic study revealed that SE had a major protein with a MW of 40.6 kDa, while protein with MW of 32.7 kDa was predominant in ASE and ATPS fractions. Pepsinogen in SE might be activated to pepsin by acidification and partitioning process. SE was quite stable at 0 °C and 4 °C up to 14 days. The loss in protease activity in ASE and selected ATPS fractions was more pronounced when storage time and temperature increased. Therefore, ATPS can be effectively used to recover and partially purify pepsin from albacore tuna stomach.

When tuna pepsin (10 units/g defatted skin) was used for collagen extraction from the skin of threadfin bream (*Nemipterus spp.*) for 12 h, the yield of collagen increased by 1.84-2.32 folds and albacore tuna pepsin showed the comparable extraction efficacy to porcine pepsin. The yield generally increased with increasing extraction time ($P < 0.05$). Collagen extraction from the skin of unicorn leatherjacket (*Aluterus monoceros*) with the aid of pepsin from albacore tuna at a level of 20 units/g skin yielded 28.33% (dry wt.), which was approximately 8-fold higher than that of acid extraction. The yields of acid solubilized collagen (ASC) and pepsin solubilized collagen (PSC), using albacore tuna pepsin, from the skin of arabesque greenling (*Pleurogrammus azonus*) were 30.3 and 14.0% (dry wt.), respectively. Based on protein patterns and TOYOPEARL® CM-650M column chromatography, both collagens contained α- and β-chains as their major components and were characterized as type I collagen. Fourier transform infrared (FTIR) spectroscopy revealed that the triple helical structure was predominant in ASC and

PSC from both species but there was a slight difference in secondary structure between both collagens. Based on ζ-potential, p*I* of collagen from both species was observed in range of 6.29-6.38.

Hydrolysis of type I collagen by different pepsins including porcine pepsin, skipjack tuna pepsin 1 and 2 was demonstrated. Purified type I collagen from the skin of ornate threadfin bream (*N. hexodon*) contained $[\alpha1(I)]_2\alpha2(I)$ as the dominant components with the co-presence of α1(I)α2(I)α3(I). After hydrolysis of purified type I collagen, the band intensity ratios of α1/α2 chain were significantly increased ($P < 0.05$) with the concomitant decrease in band intensity of higher MW cross-linked components. The cross-linked components were effectively hydrolyzed by pepsin 1 and 2 from skipjack tuna stomach and porcine pepsin at 4 °C without the cleavage of β and α-chains. At 50 °C, purified type I collagen was more susceptible to porcine pepsin hydrolysis, followed by skipjack tuna pepsin 2 and 1, respectively.

Functional properties and antioxidative activities of protein hydrolysates prepared from ornate threadfin bream muscle, using skipjack tuna pepsin, with different degree of hydrolysis (DH: 10, 20, 30%), were determined. The solubility of hydrolysates varied from 71 to 99% in the pH range of 3-9, depending on their DH. Emulsifying and foaming properties of hydrolysates were governed by DH and concentrations used. Hydrolysates with 20% DH had the highest ABTS and DPPH radical scavenging activities. However, chelating activity on Fe^{2+} of hydrolysate increased as DH increased ($P < 0.05$). Size exclusion chromatography of hydrolysates with 20% DH using Sephadex G-25 revealed that antioxidative peptides with molecular weight of approximately 1.3 kDa exhibited the highest ABTS radical scavenging activity. The fraction containing antioxidative peptides was quite stable over a wide pH range (1-10) and had high stability when heated at 100 °C for up to 3 h. *In vitro* simulated gastrointestinal digestion revealed that ABTS radical scavenging activity of the antioxidative peptides was not affected by pepsin hydrolysis, while further digestion by pancreatin enhanced the activity. Therefore, protein hydrolysates produced by skipjack tuna pepsin can be used as a promising source of functional antioxidative peptides.

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LIST OF ABBREVIATION

A_{220}	=	absorbance at 220 nm
A_{230}	=	absorbance at 230 nm
A_{280}	=	absorbance at 280 nm
ABTS	=	2,2-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt
AC	=	actin
ASC	=	acid solubilized collagen
APSE	=	partitioned acidified stomach extract
ASE	=	acidified stomach extract
ATP	=	Adenosine 5′-triphosphate
ATPS	=	aqueous two-phase system
βME	=	*β*-mercaptoethanol
BSA	=	bovine serum albumin
C	=	control
°C	=	degree Celsius
CSC	=	calf skin collagen
C-OTB	=	purified type I collagen from ornate threadfin bream skin
C-PP	=	purified type I collagen from ornate threadfin bream skin treated with porcine pepsin
C-SP1	=	purified type I collagen from ornate threadfin bream skin treated with skipjack pepsin 1
C-SP2	=	purified type I collagen from ornate threadfin bream skin treated with skipjack pepsin 2
DEAE	=	diethylaminoethyl
DH	=	degree of hydrolysis
DPPH	=	2,2-diphenyl-1-picryl hydrazyl
DSC	=	Differential scanning calorimetry
E-64	=	1-(L-trans-epoxysuccinyl-leucylamino)-4-guanidinobutane
EAI	=	emulsifying activity index

LIST OF ABBREVIATION (Continued)

EDTA	=	ethylenediaminetetraacetic acid
ESI	=	emulsifying stability index
FE	=	foam expansion
FS	=	foam stability
FTIR	=	Fourier transform infrared
g	=	gram
GI	=	gastrointestinal
h	=	hour
H	=	high molecular weight protein marker
ΔH	=	total denaturation enthalpy
HMC	=	high molecular weight cross-linked component
Hyp	=	hydroxyproline
I	=	type I collagen
k_{cat}	=	turn over number
kDa	=	kilodalton
K_m	=	Michelis-Menten rate constant
M	=	molecular weight protein marker
mg	=	miligram
MHC	=	myosin heavy chain
mL	=	milliliter
min	=	minute
μM	=	micromolar
MW	=	molecular weight
N	=	normal
nm	=	nanometer
PAGE	=	polyacrylamide gel electrophoresis
PEG	=	polyethylene glycol
PG	=	pepsinogen

LIST OF ABBREVIATION (Continued)

p*I*	=	isoelectric point
PP	=	porcine pepsin
PSC	=	pepsin solubilized collagen
PSE	=	partitioned stomach extract
rpm	=	round per minute
SP1	=	skipjack tuna pepsin 1
SP2	=	skipjack tuna pepsin 2
SB	=	starting buffer
SD	=	standard deviation
SDS	=	sodium dodecyl sulfate
SE	=	stomach extract
TCA	=	trichloroacetic acid
TE	=	Trolox equivalent
TEMED	=	*N,N,N′,N′*-tetramethylethylene diamine
T_m	=	maximum transition temperture
TM	=	tropomyosin
TNBS	=	2,4,6-trinitrobenzenesulphonic acid,
TNT	=	troponin T
Trolox	=	6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid
UV	=	ultraviolet
VMC	=	very high molecular weight cross-linked component
V_{max}	=	maximal velocity
W	=	wide range molecular weight protein marker
wt	=	weight
ζ	=	zeta

CHAPTER 1

INTRODUCTION AND REVIEW OF LITERATURE

1.1 Introduction

Thailand is the top canned tuna producer and exporter in the world (Department of Foreign Trade, 2007). By the year 2006, Thai canned tuna was exported to several countries with the total volume of 416,226 metric tons and a value of 40,408 million bahts (The Customs Department, 2007). Three tuna species, including albacore, yellowfin and skipjack, are mainly used for the production of canned tuna in Thailand (Department of Foreign Trade, 2007). During canned tuna manufacturing, about two-thirds of the whole fish are utilized and the processing wastes at 450,000 metric tons annually are generated (Subasinghe, 1996). Among those byproducts, fish viscera at a high portion are a potential source of various proteases. Pepsin is the major digestive enzyme in stomach of animals that are secreted as pepsinogen from chief cells of oxyntic glands located in the stomach wall epithelium. In acidic environment, pepsinogens rapidly convert to pepsin (Kageyama, 2002). Pepsins and pepsinogens from stomach of various fish species such as Pacific yellowfin tuna (Norris and Mathies, 1953), Arctic fish capelin (Gildberg and Raa, 1983), polar cod (Arunchalam and Haard, 1985), North Pacific bluefin tuna (Tanji *et al.*, 1988), Atlantic cod (Gildberg *et al.*, 1990), sea bream (Zhou *et al.*, 2007), African coelacanth (Tanji *et al.*, 2007), pectoral rattail (Klomklao *et al.*, 2007), smooth hound (Bougatef *et al.*, 2008), Mandarin fish (Zhou *et al.*, 2008), Pacific bluefin tuna (Tanji *et al.*, 2009), European eel (Wu *et al.*, 2009a) and snakehead (Chen *et al.*, 2009) have been purified and characterized.

Nowadays, industry demands the efficient and economical downstream processes for the partitioning and purification of biomolecules that give high yield and high purity of the product. Partitioning in aqueous two-phase system (ATPS) has been shown to be powerful for separating and purifying the mixtures of proteins (Bensch *et al.*, 2007; He *et al.*, 2005; Tanuja *et al.*, 1997). ATPS forms readily upon mixing

aqueous solutions of two hydrophilic polymers, or of a polymer and a salt, above a certain threshold concentration. In general, ATPS yields a specific environment suitable for maintaining enzymes in their native structure and for concentration/purification by means of selective partitioning of the enzyme to one of the phases (Tanuja *et al.*, 1997). Coincidentally, ATPS can remove by-products such as other undesirable enzymes/proteins, unidentified polysaccharides and pigments that are present in the system (Srinivas *et al.*, 2002; Tanuja *et al.*, 1997). Therefore, this method can be made highly selective and can be easily scaled up, thus allowing wider biotechnological applications (Chouyyok *et al.*, 2005; He *et al.*, 2005).

Pepsin has been applied for fish protein hydrolysate preparation (Liu and Pigott, 1981), production of protein hydrolysate from chicken heads (Surówka and Fik, 1994), and for preparation of gelatin from bovine skin (Chomarat *et al.*, 1994) and bigeye snapper skin (Nalinanon *et al.*, 2008a). It can be used as rennet substitute for cheese production (Brewer *et al.*, 1984). Since pepsin is able to cleave peptides in telopeptide region of collagen specifically, pepsin has been successfully used for collagen extraction with the higher yield (Jongjareonrak *et al.*, 2005; Nagai *et al.*, 2001; Nalinanon *et al.*, 2007). Due to a plenty of tuna viscera, especially stomach, the recovery of pepsin from tuna viscera with the appropriate downstream processes should be taken into consideration. Also, uses of recovered tuna pepsin for production of protein hydrolysate with neutraceutical properties should be focused to gain the novel product with increased market value. Therefore, the information gained will be beneficial for tuna processing plants as well as food and pharmaceutical industries.

1.2 Review of Literature

Pepsin and pepsinogen are the major proteinases in the digestive tracts of animals localized in the stomach. Pepsin is involved in the hydrolysis of proteins under the acidic condition in stomach. Pepsin can be found in the latent form and is activated under the physiological condition.

1.2.1 Pepsinogen

In general, the inactive pepsinogen is produced by the chief cells of the oxyntic glands, which are located in the stomach wall epithelium and secreted into the stomach (Gildberg, 1988; Whitaker, 1994). The zymogen is converted to the active pepsin in the presence of HCl.

1.2.1.1 Primary structure

Pepsinogens have been isolated and sequenced, at either the protein or the DNA level, from a wide range of vertebrates, including mammals, avians, amphibians and fish. The complete amino acid sequences of pepsinogens A from Japanese monkey (Kageyama and Takahashi, 1986; Kageyama *et al.*, 1991), pig (Lin *et al.*, 1989), chicken (Baudyš and Kostka, 1983), rabbit (Kageyama *et al.*, 1990) and turtle (Hirasawa *et al.*, 1996) were reported. Based on amino acid sequence, tuna pepsinogen 2 could be classified as pepsinogen B (Tanji *et al.*, 1996). In addition, different pepsinogens including bovine pepsinogen A (Harboe *et al.*, 1974), pig pepsinogen B (Nielsen and Foltmann, 1995), and tuna pepsinogens 1 and 3 (Tanji *et al.*, 1988) were found (Figure 1).

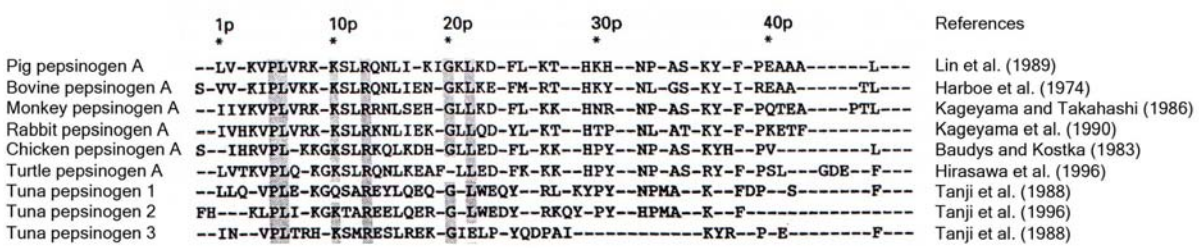

Figure 1. Alignment of the amino acid sequences of prosegments of the pepsinogens. The numbering is based on the sequences of pig pepsinogens, and begins at the N-terminus of the prosegment. The suffix 'p' denotes residues in the prosegment. The highly conserved residues at positions 5p (Pro), 6p (Leu), 10p (Lys), 13p (Arg), 20p (Gly) and 22p (Leu) are highlighted.

Source: Richter *et al.* (1998) with a slight modification.

The pesinogens consist of single polypeptide chains of approximately 370 amino acids. As indicated in Table 1, the prosegment moieties at the N-terminus of the zymogens range in length from 35 to 47 amino acid residues and are highly basic. In contrast, the active enzyme moieties contain a large number of Asp and Glu residues and are highly negatively charged (Richter *et al.*, 1998). The prosegments of the gastric zymogens show considerable identity (Figure 1). More significantly, residues Pro-5p, Leu-6p, Lys-10p, Arg-13p, Gly-20p and Leu-22p are conserved in nearly all proteinases whose sequences are known. The numbering of the amino acid residues is based on the sequence of pig pepsinogen A, and the suffix 'p' is used to denote residues in the prosegment. Non-polar residues are present at positions lp, 4p, 12p, 16p, 22p, 25p and 37p, basic residues at positions 3p, 8p and 36p, and hydroxylated residues at position 11p. Differences in the sequences of the prosegments are most notable at the C-terminus, in the region connecting the prosegment to the active enzyme moiety (Richter *et al.*, 1998). The conserved non-polar amino acids at positions 12p, 16p, 22p and 25p form the hydrophobic core of the prosegment. The conserved basic residues at positions 3p, 8p, 10p, lap and 36p are engaged in electrostatic interactions, and the conserved hydroxylated residue at position 11p and the conserved Gly at 20p cap the N- and C-terminus, respectively of the first helix of the prosegment (Richter *et al.*, 1998).

Table 1. Amino acid composition of pepsinogens

Enzyme	No. of residues			Refs.
	Total	Prosegment	Basic residue in prosegment	
Monkey pepsinogen A	373	47	12	(a)
Bovine pepsinogen A	ND*	45	12	(b)
Pig pepsinogen A	370	44	13	(c)
Pig pepsinogen B	ND	43	11	(d)
Rabbit pepsinogen A	372	44	12	(e)
Chicken pepsinogen A	367	42	14	(f)
Turtle pepsinogen A	361	46	10	(g)
Tuna pepsinogen 1	ND	41	5	(h)
Tuna pepsinogen 2	360	37	10	(i)
Tuna pepsinogen 3	ND	35	8	(h)

*ND = not determined.

Sources: (a) Kageyama and Takahashi (1986), (b) Harboe *et al*. (1974), (c) Lin *et al*. (1989), (d) Nielsen and Foltmann (1995), (e) Kageyama *et al*. (1990), (f) Baudyš and Kostka (1983), (g) Hirasawa *et al*. (1996), (h) Tanji *et al*. (1988) and (i) Tanji *et al*. (1996).

1.2.1.2 Tertiary structure

Three-dimensional structures have been determined for porcine pepsinogen A (Hartsuck *et al.*, 1992; Sielecki *et al.*, 1991) and pepsin A (Abad-Zapatero *et al.*, 1990; Andreeva *et al.*, 1984; Cooper *et al.*, 1990; Sielecki *et al.*, 1991), human pepsinogen A (Bateman *et al.*, 1998) and pepsin (Fujinaga *et al.*, 1995). X-ray crystallographic data revealed the tertiary structures of the active enzyme moieties of these zymogens to be very similar and common to those of other enzymes belonging to the aspartic proteinase family (Kageyama, 2002). Pepsins consist largely of β sheets and the fold of the protein is stabilized by a large number of hydrogen bonds. Pepsins and other aspartic proteinases share bilobal symmetry, and, in the case of porcine pepsin A, residues 1–175 form the N-terminal lobe, while residues 176 to 326 constitute the C-terminal lobe (Cooper *et al.*, 1990).

1.2.1.3 Conversion of pepsinogen to pepsin

Pepsin, as well as other gastric proteases, are synthesized and secreted as zymogens which are converted into active enzymes in the acidic gastric juice. This process involves a series of conformational changes and bond cleavage, resulting in the unveiling of the active site and ultimate removal and dissociation of the prosegment from the active center of the enzyme (Richter *et al.*, 1998). At pH 2, the reaction is rapid and autodigestion does not occur during the activation time. The intramolecular reaction which predominates at pH 2 produces cleavage at a single point, whereas autocatalytic activation could generate several NH_2 termini (Al-Janabi *et al.*, 1972). The reaction then proceeds by limited proteolysis that finally removes 44 amino acid residues (the prosegment) from the N-terminal end of the zymogen peptide chain (Nielsen and Foltmann, 1993). Zhou *et al.* (2007) found that four pepsinogens (PG-I, PG-II, PG-III, and PG-IV) purified from sea bream (*Sparus latus* Houttuyn) stomach converted into pepsin within a few minutes at pH 2.0. Pepsinogen with concentrations less than 0.2 mg/ml was cleaved between Leul6p and Ile17p at pH 2 (Christensen *et al.*, 1977). At concentrations of pepsinogen above 0.5 mg/ml and pH 2, the Leu44p-Ile1 bond is predominantly cleaved directly (Kageyama and Takahashi, 1983). Nielsen and Foltmann (1995) reported that activation of porcine pepsinogen B at pH 2 resulted in formation of the covalent intermediate (pseudo-pepsin B) by proteolytic cleavage of bond Met16p-Glu17p. Pseudo-pepsin B was stable at pH 2 and readily converted to pepsin B at pH 5.5. During the conversion of pepsinogen B to mature pepsin B, a segment of 43 amino acid residues was cleaved from N-terminal of pepsinogen B (Nielsen and Foltmann, 1995). In pepsinogen, the prosegment covers the active-site cleft, and at neutral or weakly alkaline conditions the inactive zymogen is stabilized through electrostatic interactions between basic amino acid residues in the prosegment and acidic residues in the enzyme moiety of the molecule (Nielsen and Foltmann, 1993). The side chain of Lys36p points down in the active-site cleft where its ε-amino group forms salt bridges to Asp32 and Asp215 (Nielsen and Foltmann, 1993). At low pH, the electrostatic interactions are weakened so that the prosegment moves out of the active-site cleft and the zymogen is converted to an active conformation. James and Sielecki (1986) suggested two possible pathways of conformational change during the activation: one in which an exchange

of the N-terminal residues occurs after proteolytic cleavage, and one in which an exchange of the N-termini in the β sheet occurs before proteolysis. Furthermore, Tanji *et al.* (1988) found the differences in activation profile of three pepsinogens from North Pacific bluefin tuna, at pH 2.0 and 14°C. Pepsinogen 1 was converted to pepsin 1 in a stepwise manner, via four intermediate forms. The generation of the first intermediate form was rapid and completed within a few seconds. The generation of the successive intermediate forms and of pepsin 1 was slower. Complete conversion of pepsinogen 1 to pepsin 1 took over 1 h. Pepsinogen 2 was converted to pepsin 2 by a two-step process through an intermediate form. The generation of the intermediate form was as rapid as that of the first intermediate form from pepsinogen 1. Pepsinogen 3 was converted directly to pepsin 3 with a slow rate and a few hours were needed for complete activation. Tanji *et al.* (2007) reported that when incubated pepsinogens from African coelacanth (*Latimeria chalumnae*) at pH 2.0, PG1 and PG2 were converted autocatalytically to the mature pepsins through an intermediate form, whereas PG3 was converted to an intermediate form, but not to the mature pepsin autocatalytically. Chen *et al.* (2009) found that the intermediate form of PG1 and PG2 from snakehead (*Channa argus*) can be observed during acid activation (pH 2.0, 37 °C), whereas the conversion of PG3 to P3 was performed by a direct pathway. Time-course study of the activation of pepsinogens from snakehead showed that active form of PG-I can be identified at a short time as 5 s, while active pepsins of PG-II and PG-III were detected after 1 min (Wu *et al.*, 2009a). Bougatef *et al.* (2008) found that complete activation of pepsinogen from smooth hound was carried out in 30 min. The conversion of pepsinogens to pepsins is expected to have a decrease in molecular mass of approximately 5 kDa because of the loss of the N-terminal propart (Chen *et al.*, 2009).

Pepsinogen has a molecular weight of 42,000 dalton and contains three disulfide bonds. It has an isoelectric point of 3.7. It is quite stable at pH 7 to 9, but at low pH it is rapidly converted to pepsin (Whitaker, 1994). Six peptide bonds are hydrolyzed in the initial conversion of pepsinogen to pepsin (Figure 2). The large peptide labeled B remains attached by noncovalent bonds to pepsin at higher pH values and is inhibitory. It readily dissociates from pepsin at pH 1 to 2 (Whitaker, 1994). Pepsin continues to act on the last large peptide, B, to produce three more bond

cleavages (bonds cleaved indicated by lower case p). Pepsin is produced only when the bond (double-underlined P in Figure 2) is hydrolyzed.

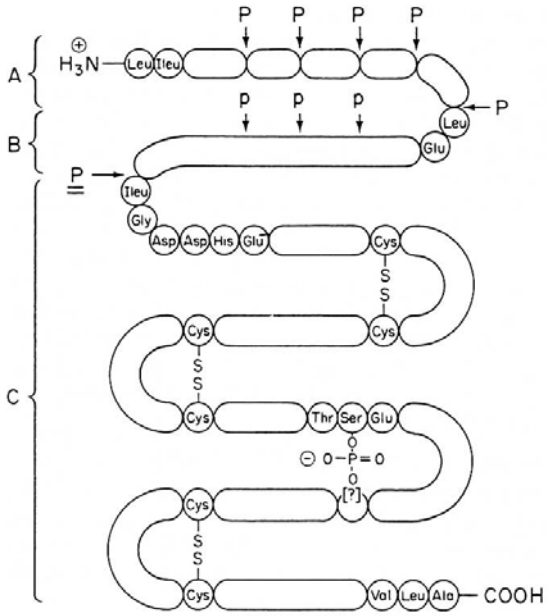

Figure 2. Schematic representations of the structure of pepsinogen and its conversion to pepsin. The major points of hydrolysis are marked with P and result in release of several peptides (A), pepsin inhibitor (B), and pepsin (C). Hydrolysis of the bond P is essential for activation.

Source: Whitaker (1994)

Pepsin is quite resistant to further proteolysis in acid solution, but at pH 5 to 7 it is rapidly inactivated, in contrast to pepsinogen (Gildberg, 1988). During conversion of pepsinogen to pepsin, the molecular weight decreases from 42,000 to 35,500 dalton and the isoelectric point changes from 3.7 to less than 1 (Whitaker, 1994). Furthermore, pepsinogen from swine has 363 amino acid residues, whereas pepsin consists of 321 amino acid residues. In the activation process, 42 residues, including 9 of lysine, 2 of histidine, and 2 of arginine, are cleaved off (Rajagopalan *et al.*, 1966). The decrease in isoelectric point is due to loss of 9 of the 10 lysyl residues, 2 of the 3 histidyl residues, and 2 of the 4 arginyl residues during activation (Whitaker, 1994). Bohak (1973) found that the conversion of chicken pepsinogen to

chicken pepsin was observed in pH range of 2-4. In this pH range, the zymogen undergoes a rapid conformational change to form an intermediate, and the subsequent conversion of this intermediate to the active enzyme is the rate-determining step (Bohak, 1973). The activation of bovine pepsinogen was also reported by Harboe *et al*. (1974). Bovine pepsinogen is converted into pepsin by removal of 45 amino acid residues from the NH_2 terminus of the single polypeptide chain. The complete sequence of the activation peptides has been deduced from two overlapping sets of peptides, one set obtained by autoactivation of the zymogen at pH 2 and the second set from a tryptic digest of maleylated pepsinogen. A peptide derived from the 17 residues at the NH_2 terminus of bovine pepsinogen was identified as an inhibitor of the milk-clotting action of pepsin (Harboe *et al.*, 1974).

1.2.2 Pepsin

Pepsin is a gastric aspartic proteinase, a class of endopeptidases active at acidic conditions, which plays an integral role in the digestion process of vertebrates (Gildberg, 1988; Tello-Solís and Romero-García, 2001). Pepsin is composed of a single polypeptide chain of 321 amino acids and has a molecular weight of 35,500 dalton. Its tertiary structure is stabilized in part by three disulfide bridges and a phosphate linkage. A phosphate group, attached to the hydroxyl group of a seryl residue, can be removed without loss of enzymatic activity (Whitaker, 1994). The enzyme is quite stable from pH 2 to about 5, but above pH 5 it rapidly loses activity due to the denaturation. The pH optimum of pepsin on proteins is about pH 2, but on synthetic substrates it is around pH 4.0 (Whitaker, 1994). Pepsin has its primary specificity toward the amino acid residue that furnishes the NH group to the susceptible peptide bond, preferably phenylalanyl, tyrosyl, or tryptophanyl. Pepsin and other aspartic proteases readily catalyze transpeptidation in the presence of suitable reactants (Whitaker, 1994).

1.2.2.1 Extraction and purification of pepsin and its zymogen

Pepsins and pepsin-like enzymes could be prepared from the digestive tracts of sea water and fresh water fish such as Atlantic cod (*Gadus morhua*) (Brewer

et al., 1984), capelin (*Mallotus villosus*) (Gildberg and Raa, 1983), Greenland cod (*Gadus ogac*) (Squires *et al.*, 1986), polar cod (*Boreogadus saida*) (Arunchalam and Haard, 1985), salmon (*Oncorhynchus keta*) (Sánchez-Chiang *et al.*, 1987), Pacific yellowfin tuna (*Thunnus albacares*) (Norris and Mathies, 1953), North Pacific bluefin tuna (*Thunnus thynuus orientalis*) (Tanji *et al.*, 1988), sardine (*Sardinos melanostica*) (Noda and Murakami, 1981), sea bream (*Sparus latus* Houttuyn) (Zhou *et al.*, 2007), African coelacanth (*Latimeria chalumnae*) (Tanji *et al.*, 2007), pectoral rattail (*Coryphaenoides pectoralis*) (Klomklao *et al.*, 2007), smooth hound (*Mustelus mustelus*) (Bougatef *et al.*, 2008), Mandarin fish (*Siniperca chuatsi*) (Zhou *et al.*, 2008), European eel (*Anguilla anguilla*) (Wu *et al.*, 2009a) and snakehead (*Channa argus*) (Chen *et al.*, 2009). Arunchalarn and Haard (1985) purified pepsin A and B from the stomachs of polar cod by homogenizing the tissue in phosphate buffer (pH 7.3), followed by filtration through a cheesecloth. The filtrate was further clarified by centrifugation and then subjected to affinity chromatography using a CBZ-D-phenylalanine-TETA-Sepharose 4B gel. Two pepsin isozymes, pepsin I and II, were also prepared from the stomach of capelin by homogenizing the tissues in cold 1 mM HCl, followed by ammonium sulfate fractionation (30-70% saturation), DEAE-cellulose, and Sephadex G-75 (Gildberg and Raa, 1983). The purified pepsins had the molecular mass of about 25,000 dalton. Pepsin was also isolated in the zymogen form from the stomach tissue of rainbow trout by homogenizing the tissue in phosphate buffer (pH 7.1), followed by centrifugation to clarify the homogenate, ammonium sulfate fractionation (20-50% saturation) of the clear extract, and then a series of chromatographic steps involving polylysine-Sepharose, DEAE-Sephacel, Sephadex G-200, and DEAE-Sepharose gels (Twining *et al.*, 1983). Gildberg *et al*. (1990) purified pepsin from Atlantic cod (*Gadus morhua*) stomach by ammonium sulfate fractionation (20-70% saturation), followed by ion exchange chromatography using S-Sepharose Fast Flow. Sánchez-Chiang *et al.* (1987) prepared pepsin from the stomach of adult and juvenile salmon by ammonium sulfate fractionation (0-70% saturation), followed by DEAE-cellulose column to separate pepsin I and II. Pepsin I was then purified by gel filtration on a Sephadex G-100 column. Purification of three pepsinogens (pepsinogen 1, 2 and 3) from gastric mucosa of the North Pacific bluefin tuna (*Thunnus thynuus orientalis*) was achieved by homogenizing the tissue in

phosphate buffer (pH 7.0) containing phenylmethanesulfonyl fluoride (2 mM), followed by ammonium sulfate precipitation (10-35% saturation), and then a series of chromatographic steps involving DEAE-cellulose, Sephadex G-150 gel filtration, Q-Sepharose Fast Flow and Mono Q HR5/5 (Tanji *et al.*, 1988). Pavlisko *et al.* (1997) purified pepsin from the stomach of palometa (*Parona signata*) by ammonium sulfate fractionation (23-63% saturation) and Sephadex G-100 column which appeared as two bands on SDS-PAGE. Four pepsinogens (PG-I, PG-II, PG-III and PG-IV) were identified in sea bream (*Sparus latus* Houttuyn) stomach and were purified to homogeneity by ammonium sulfate fractionation (20-60% saturation) and subsequent chromatographies including DEAE-Sephacel and Sephacryl S-200 HR, respectively (Zhou *et al.*, 2007). Two pepsins (A and B) were purified from the stomach of pectoral rattail (*Coryphaenoides pectoralis*) by acidification, ammonium sulfate precipitation (30-70% saturation), and then a series of chromatographic steps involving Sephacryl S-200, DEAE-cellulose and Sephadex G-50 (Klomklao *et al.*, 2007). Recently, three pepsinogens (PG-I, PG-II and PG-III) were purified to homogeneity from the stomach of European eel (*Anguilla anguilla*) by ammonium sulphate fractionation (20-60% saturation) and subsequent chromatographies including DEAE-Sephacel and Sephacryl S-200 and Superdex G-75, respectively (Wu *et al.*, 2009a).

The purification of pepsins and their zymogens from mammals and other vertebrates have also been investigated. Chow and Kassel (1968) purified pepsinogen from bovine mucosa of the fourth stomach (abomasum) by ammonium sulfate fractionation (23-63% saturation), batch absorption on DEAE-cellulose, recycling gel filtration on Sephadex G-100, and finally chromatography on DEAE-cellulose. Sheep pepsin was isolated (approx. 120-fold purification) from aqueous abomasal homogenates by acidification (pH 2.0), affinity chromatography on Sepharose 4B-poly-L-lysine columns and gel filtration on Sephadex G-100 (Fox *et al.*, 1977). Kageyama and Takahashi (1980) purified eight pepsinogens (namely pepsinogen I-a, I-b, III-1-a, III-1-b, III-2-a, III-2-b, III-3 and C) from gastric mucosa of the crab-eating monkey (*Macaca fascicularis*) by DE-32 cellulose chromatography, Sephadex G-150, DE-32 rechromatography and electrophoretic separation. A pepsinogen from dog (*Canis familliaris*) stomach has been purified by two steps of

centrifugation (27000*g* and 90000*g*, respectively), DEAE-Sephacel column, an Amicon YM-10 membrane concentrating prior to adsorb onto a polylysine-Sepharose column and finally separated onto Sephadex G-200 column (Twining *et al.*, 1983). Baudyš *et al.* (1988) purified pepsinogen A from the stomach of Mongolian lamb (*Ovis platyurea*) to homogeneity by salt precipitation (using $NH_4Al(SO_4)_2$ (0.33 M) and ammonium sulfate precipitation (95% saturation)), followed by a series of chromatographies involving DEAE-cellulose, DEAE-Sephadex A-50 and Sephadex G-100. Three type-A and two type-C pepsinogens, namely, pepsinogens A-1, A-2, A-3, C-1, and C-2 were purified from adult goat (*Capra hircus*) abomasums by a series of chromatographies including DEAE-Sephacel, Sephadex G-100 and FPLC on a mono Q column HR 5/5 (Suzuki *et al.*, 1999). Purification of porcine pepsinogen B was carried out using DEAE-cellulose (DE32), Sephacryl S-100 and FPLC on a Mono Q HR 5/5 (Nielsen and Foltmann, 1995). Pichová and Kostka (1990) prepared pepsinogen from duck (*Anas platyrhynchos*) glandular stomach by ammonium sulfate precipitation (45-80% saturation), DEAE-cellulose and finally by FPLC using a Mono Q HR 5.5. For duck pepsin, acidification of pepsinogen was achieved at pH 1.8 and the activation peptides were separated on Sephadex G-100, followed by the affinity chromatography on Val-D-Lue-Pro-Phe-Val-D-Leu Sepharose 4B. Pepsinogen from another avian has been isolated. Streicher *et al.* (1985) isolated pepsinogens from the proventriculus of the ostrich (*Struthio camelus*) by homogenizing the proventriculi in Tris buffer (pH 7.5), followed by filtration through a cheesecloth. The filtrate was further clarified by centrifugation and then subjected to a series of chromatographies involving DEAE-cellulose, Sephadex G-100 and Hydroxylapatite column. Furthermore, pepsinogens and pepsins from the esophageal mucosa of bullfrog (*Rana catesbeiana*), an amphibian species, have also been purified by ammonium sulfate precipitation (5-35% saturation), DEAE-cellulose, Sephacryl S-200 gel filtration and Q-Sepharose fast flow column chromatography (Yakabe *et al.*, 1991).

Isolation of pepsin or pepsinogen by affinity chromatography using specific immobilized ligands for the enzyme has been paid much attention. Arnoštová *et al.* (2001) prepared the affinity sorbents containing immobilized iodinated derivatives of L-tyrosine for the affinity chromatography of porcine pepsin. The ligand was coupled either to Sepharose 4B or bead cellulose after the divinylsulfone

activation or to Sepharose 4B after the activation with 2,4,6-trichloro-1,3,5-triazine. The highest capacity to recover porcine pepsin (95%) was found when 3,5-diiodo-L-tyrosine coupled to divinylsulfone-activated Sepharose was used. Frýdlová *et al*. (2004) developed the affinity chromatography of porcine protease and its zymogen by using immobilized substrate specific for pepsin. For the immobilization of *N*-acetyl-L-phenylalanine and 3,5-diiodo-L-tyrosine, divinyl sulfone activated Sepharose was used. Ligands with blocked amino group and free carboxyl one were linked to Sepharose via ethylene diamine spacer using carbodiimide reaction. The recovery of loaded enzyme to both affinity carriers was approximately 96% for *N*-acetyl-L-phenylalanine-Sepharose and 88% for immobilized 3,5-diiodo-L-tyrosine. Pepsin was adsorbed to both immobilized ligands at pH 3.5–4.0. To elute the enzyme, it was necessary to increase ionic strength (up to 0.5 M). For the adsorption of pepsinogen, pH of 5.2 was found to be optimum and an increase of ionic strength was used for its desorption (Frýdlová *et al.*, 2004). Furthermore, stationary phase containing quinolin-8-ol immobilized on macroporous methacrylate support for the affinity chromatography of porcine pepsin A was described by Novotná *et al.* (2005). Optimized chromatographic conditions for separation of porcine pepsin A on this stationary phase were influenced by pH, concentration, ionic strength and chemical composition of the used mobile phases. The stationary phase showed a good reproducibility, high recovery and a satisfactory capacity for porcine pepsin A.

1.2.2.2 Catalytic mechanism of pepsin

A proposed mechanism for the action of pepsin, involving nucleophilic catalysis, is shown in Figure 3. The free enzyme has two carboxyl groups, one in the protonated form and the other in the ionized form, in the transforming locus of the active site. The enzyme-substrate adsorptive complex is formed, followed by a nucleophilic attack of the carboxylate group on the carbonyl group of the peptide bond. This leads to the formation of a covalent tetrahedral intermediate (Whitaker, 1994).

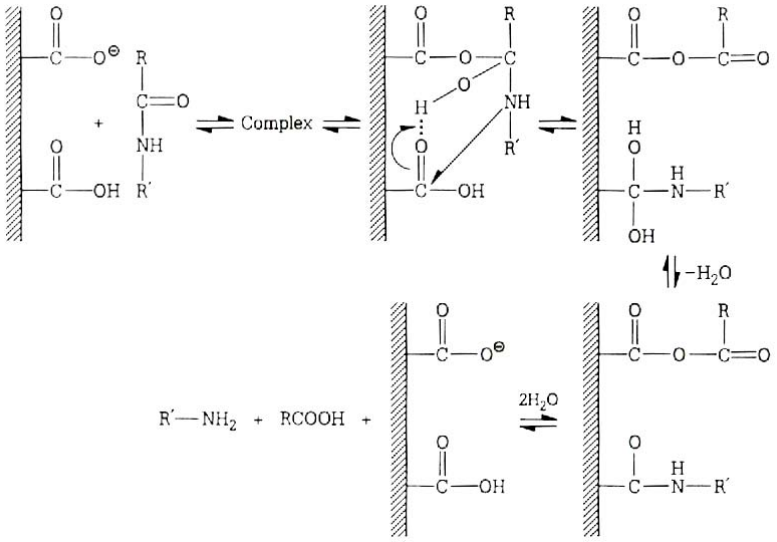

Figure 3. Catalytic mechanism of pepsin
Source: Whitaker (1994)

The carbonyl oxygen of the protonated carboxyl group extracts a proton from the hydroxyl group, facilitating an electrophilic attack of the carbonyl carbon on the NH group of the peptide bond. The result is the formation of an aminoacylenzyme intermediate, which then reacts with water to give the products of the reaction (Whitaker, 1994).

1.2.2.3 Pepsin inhibitors

Diazoacetyl norleucine methylester (DAN) was the first inhibitor used for specific, covalent inhibition of aspartic proteinases (Fusek and Větvička, 1995). Rajagopalan *et al.* (1966) revealed that DAN rapidly inactivates pepsin. In the presence of Cu^{2+} ions, one of the two aspartates within the active site of pepsin reacts with DAN. A single carboxyl group at the active site of pepsin may have been esterified by this reagent. Several substrate-like epoxides were found by Tang (1971) to act as specific and irreversible inactivators of pepsin. Of these, 1,2-epoxy-3-(*p*-nitrophenoxy) propane (EPNP) was the most potent. When EPNP reacted with pepsin, all enzyme activity was lost. Two molecules of EPNP were found to be covalently bound to each molecule of completely inactivated enzyme (Tang, 1971).

Some naturally occurring inhibitors are known in addition to synthetic inhibitors (Kageyama, 2002). The most potent is pepstatin, isolated from *Streptomyces* species, which contains two residues of a novel amino acid, statine, and strongly inhibits pepsin A with a K_i value of 4.6 x 10^{-11} M (Rich and Sun, 1980). The inhibitor binds to an extended conformation in the active-site cleft, and the first statyl hydroxyl oxygen interacts strongly with the catalytic aspartate via hydrogen bonding to the essential carboxyl groups (Davies, 1990). Due to the extremely high affinity of pepstatin, the activity of pepsin A is inhibited completely in the presence of equimolar amounts. Pepstatin is effective against aspartic proteinases in general, although its affinity differs between enzymes. Gastricsin and chymosin are less susceptible, and about 100- and 10-fold molar excesses, respectively, are needed for complete inhibition (Kageyama, 2000). Marciniszyn Jr. *et al.* (1976) prepared four derivatives of pepstatin, each of which contains the unusual amino acid 4-amino-3-hydroxy-6-methylheptanoic acid (statine). All four were potent porcine pepsin inhibitors. Furthermore, other acid proteases, human pepsin, human gastricsin, renin, cathepsin D, the acid protease from *Rhizopus chinensis* and bovine chymosin, were also inhibited by pepstatin and its derivatives (Marciniszyn Jr. *et al.*, 1976). It is suggested that the statyl residue is responsible for the unusual inhibitory capability of pepstatin and that statine is an analog of the previously proposed transition state for catalysis by pepsin and other acid proteases.

Abu Erreish and Peanasky (1974b) found that *Ascaris* pepsin inhibitors isolated from body wall of *Ascaris lumbricoides* inhibited porcine, bovine and human pepsins, and porcine gastricsin, but not human gastricsin. Each inhibitor forms a stoichiometric complex with human pepsin and with porcine gastricsin but does not react with human gastricsin. All of the inhibitors delayed milk-clotting activity of porcine pepsin at pH 5.3. The pepsin-inhibitor complex forms between pH 2 and 5 in less than 20 s and is completely dissociated in 30 min at pH 8.8 and 37 °C (Abu Erreish and Peanasky, 1974a). Mathialagan and Hansen (1996) found that serpins from the uterine of ovine can bind specifically to immobilized pepsin A and can weakly inhibit the proteolytic activities of pepsin A and C (but not cathepsins D and E). Abuereish (1998) isolated a pepsin inhibitor from the aqueous extracts of the

roots of *Anchusa strigosa.* The extract of 1 g dry roots inhibited 9,380 ± 390 µg of pepsin. The inhibitor inhibited peptic milk-clotting activity at pH 5.3.

1.2.2.4 Fish pepsin

The pepsins from fish and marine animals have molecular weights ranging from 27 to 42 kDa (Arunchalam and Haard, 1985; Gildberg and Raa, 1983; Noda and Murakami, 1981; Squires *et al.*, 1986; Tanji *et al.*, 1988; Zhou *et al.*, 2007). They had relatively higher pH optima for the hydrolysis of their substrates than did mammalian pepsins, and were generally more stable at relatively higher pH values (pH 2.0-4.0) (Arunchalam and Haard, 1985; Gildberg and Raa, 1983; Noda and Murakami, 1981; Squires *et al.*, 1986). While pepsins are unstable under alkaline conditions, their zymogens (pepsinogens) may be stable under these conditions. Pepsins from the digestive glands of marine animals are quite resistant to autolysis at low pH (Raa, 1990). However, marine pepsins were inhibited by pepstatin in a similar fashion to mammalian pepsin (Noda and Murakami, 1981; Sánchez-Chiang *et al.*, 1987). Pepsins from marine animals display a wide temperature optima range for the hydrolysis of their substrates (37-55 °C). In general, pepsins from colder-water fish had lower temperature optima and were more heat-labile than those of animals from the warmer environment (Arunchalam and Haard, 1985; Gildberg and Raa, 1983; Squires *et al.*, 1986).

1.2.2.4.1 Optimal pH

Fish pepsins usually have higher optimum pH than mammalian pepsin (Gildberg, 1988; Kubota and Ohnuma, 1970). The purified pepsin from rainbow trout had an optimum pH of 3 (Twining *et al.*, 1983). Stomach extracts from a number of animals, including fish, give two separate pH optima for protein digestion at acidic condition (Gildberg and Raa, 1983; Owen and Wiggs, 1971). Another proposed explanation to this phenomenon was that fish pepsins had two different pH optima, one in strong acid conditions, resembling mammalian pepsins, and another at about pH 3 (Norris and Mathies, 1953). Many fishes secrete at least two pepsins with different pH optima (Gildberg and Raa, 1983; Noda and Murakami, 1981). These pepsins are usually referred to as Pepsin I and Pepsin II. Pepsin I has pH optimum for

hemoglobin digestion in the pH range of 3-4, whereas Pepsin II is most active in the pH range of 2-3. The maximal activity of pepsin I and II from capelin was observed at pHs of 3.7 and 2.5, respectively, and temperature of 38 and 43 °C, respectively, when hemoglobin was used as a substrate (Gildberg and Raa, 1983). Tanji *et al*. (1988) reported the optimal pH values of pepsins 1, 2 and 3 from North Pacific bluefin for hemoglobin digestion were 2.9, 2.4 and 2.4, respectively, which were higher than that obtained for porcine pepsin A. The specific activity of pepsin 2 was two to five times higher than that of the other two, when measured under the standard conditions. Some fishes feeding on algae have very high gastric HCl secretion and pepsins with pH optimum close to 2 (Moriarty, 1973). In capelin and cod, the isoelectric properties of the two pepsins are very different. The pI of pepsin I was in the range of 6.5-7, whereas pepsin II had pI close to 4 (Gildberg and Raa, 1983; Gildberg *et al.*, 1990). The pH profiles of activity of pepsins 1 and 2 from African coelacanth toward acid denatured hemoglobin are compared with that of porcine pepsin A, typical A-type pepsin (Tanji *et al.*, 2007). The optimum pH is approximately 2.0 to 2.1 for pepsin 1 and 2.3 for pepsin 2, which is slightly higher than that of porcine pepsin. Both pepsins showed around 30% of the optimal activity at pH 4.5, whereas porcine pepsin A has less than 4% of the optimal activity. Even at pH 5.0, both pepsins appear to have some activity.

1.2.2.4.2 Optimal temperature

Pepsins from cold and temperate water fish have comparatively high activity at low temperatures (Gildberg and Raa, 1983), and they express a much lower temperature coefficient than mammalian pepsins (Brewer *et al.*, 1984). Fish pepsins seem to have lower activation energies than mammalian pepsins. Also, pepsin from cold habitat fish had lower activation energies than pepsin from fish of the same species acclimated to higher temperatures (Haard *et al.*, 1981). Temperature optimum of American salmon pepsins increased from about 32 °C at pH 1.9 to about 37 °C at pH 3.0, when hemoglobin was used as a substrate (Haard *et al.*, 1981). The temperature optimum of pig pepsin was about 20 °C higher than that of capelin pepsins (Gildberg and Raa, 1983; Gildberg, 1987). However, there are only minor differences between temperature optima of pepsins from cold and warm water fish.

Temperature optimum of stomach extracts from Pacific yellowfin tuna was about 42 °C (Norris and Mathies, 1953). Two pepsins (A and B) from the stomach of pectoral rattail showed the same optimal temperature at 45 °C (Klomklao *et al.*, 2007). A temperature optimum of 35 °C was detected for bonito pepsin (Kubota and Ohnuma, 1970), and 37 °C for polar cod (Arunchalam and Haard, 1985), whereas different temperature optima (40 and 55 °C) were found for two pepsins from sardine (Noda and Murakami, 1981). The optimum temperatures of four pepsins from sea bream were also observed in the range of 45-50 °C (Zhou *et al.*, 2007).

1.2.2.4.3 Catalytic activity

Fish pepsins have very low activity on small peptide substrates (Kubota and Ohnuma, 1970; Noda and Murakami, 1981; Sánchez-Chiang *et al.*, 1987), and a hexapeptide is the smallest substrate reported to be susceptible to hydrolysis by fish pepsin (Guerard and Le Gal, 1987). Like other aspartic proteinases, fish pepsins are very active on hemoglobin. Pepsins hydrolyze hemoglobin 3-10 times faster than casein and myofibrillar proteins from fish (Gildberg and Raa, 1983; Squires *et al.*, 1986). Twining *et al*. (1983) found that at pH 2, rainbow trout pepsin was almost 5 times as active against a hemoglobin substrate as was pig pepsin. The pH optimum of Greenland cod protease I was at pH 3.5 with hemoglobin as the substrate and was at pH 3.0 with casein as the substrate (Squires *et al.*, 1986). Moreover, Sanchez-Chiang *et al*. (1987) found that adult salmon (*Oncorhynchus keta*) consists of pepsin I and pepsin II, while only pepsin II was isolated from juvenile salmon. In contrast with pepsin II, pepsin I was activated by NaCl. However, the proteolytic activities of pepsins from the stomach of pectoral rattail was continuously decreased as NaCl concentration increased (0-30%) (Klomklao *et al.*, 2007). Gildberg *et al.* (1990) also reported that all pepsins from Atlantic cod were strongly depressed by the presence of NaCl in the concentrations of 5 and 10%; however, pepsin I was apparently less inhibited than cathepsin D.

The cleavage specificity of pepsin toward oxidized insulin B chain is unique (Figure 4). Tanji *et al.* (2007) revealed that both pepsins 1 and 2 from African coelacanth cleaved the Leu15–Tyr16 bond most rapidly. No cleavage occurred at the

adjacent Ala14–Leu15 and Tyr16–Leu17 bonds, which are usually cleaved simultaneously, but more slowly, with the Leu–Tyr bond by tetrapod pepsins A and C. It is interesting to note that upon activation of PG1 and PG2, two Leu–Ala bonds were cleaved, consistent with the cleavage specificity of the enzyme observed with oxidized insulin B chain. Thus, coelacanth pepsins 1 and 2 are thought to have primary preference for Leu at the P1 position of the substrates. In addition, the Phe25–Tyr26 bond was cleaved at a moderate rate, and the cleavage of Phe24–Phe25 bond was very slow. Thus, the specificity somewhat resembles that of mammalian pepsin A rather than that of mammalian pepsin C. Type of specificity on oxidized insulin B chain was reported for shark pepsin (Nguyen *et al.*, 1998), which cleaves the Leu15–Tyr16 bond rapidly, followed by slower cleavages at the Phe24–Phe25, Phe25–Tyr26, and Leu11–Val12 bonds.

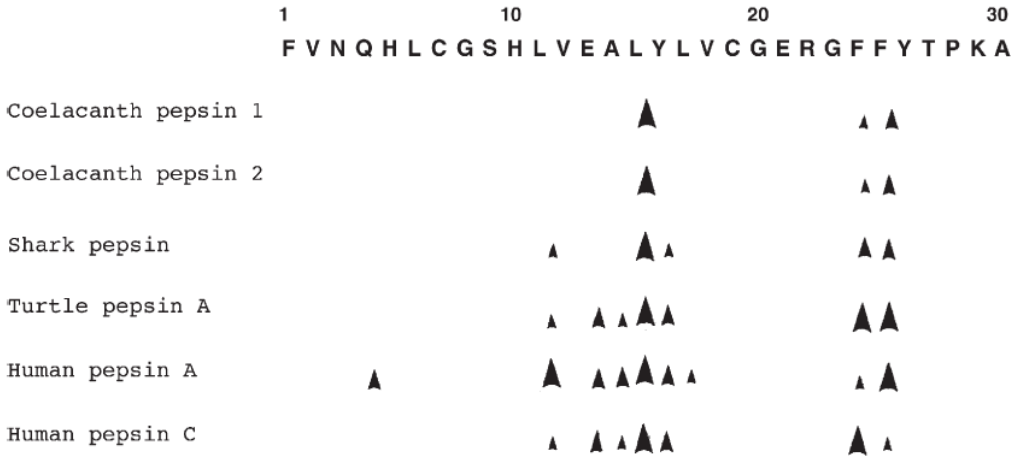

Figure 4. Cleavage of oxidized insulin B chain by different pepsins. Large arrowhead, rapid cleavage; medium arrowhead, moderate cleavage; small arrowhead, slow cleavage.

Source: Tanji *et al*. (2007)

1.2.2.4.4 Kinetic properties

Atlantic cod pepsin exhibited a relatively high and temperature dependent Michaelis constant. The minimal K_m was observed at a temperature near 0 °C. The average temperature coefficient Q_{10} for the hydrolysis of hemoglobin between 0 and 30 °C was approximately 1.4 (Brewer *et al.*, 1984). Both polar cod

pepsin isozymes from the gastric mucosa showed a lower activation energy, Ea, and higher K_m' compared with pepsin from fishes living in temperate or warm waters (Arunchalam and Haard, 1985). The relatively higher Km value obtained for pepsin B indicated that this isozyme contributed more to protein digestion at high substrate concentration than pepsin A, which would be expected to be a more efficient catalyst at low substrate concentration. Gildberg *et al.* (1990) found that Atlantic cod pepsin isozymes have higher K_m than mammalian pepsins at several assay temperatures. For each isozyme, the lowest K_m' was obtained at 10 °C. The K_m' of pepsin I was considerably higher, indicating that this enzyme was most important during late stages of digestion where the amount of soluble substrate is high. Both pepsins I and II had higher substrate affinity and greater physiological efficiency at pH 3.5 than at pH 2.0 for the hydrolysis of hemoglobin (Gildberg *et al.*, 1990).

1.2.2.4.5 Effect of inhibitors and some chemicals

effect of various inhibitors on pepsins from several fish species has been studied. Klomklao *et al*. (2007) reported that the activities of both pepsins A and B from pectoral rattail stomach were almost completely inhibited by pepstatin A (aspartic proteinases inhibitor). Tanji *et al*. (2007) also reported that both pepsins from African coelacanth were nearly completely inhibited by an equimolar amount of pepstatin. The same results were also observed in pepsins from the stomach of several fishes such as Arctic fish capelin (Gildberg and Raa, 1983), Atlantic cod (Gildberg *et al.*, 1990) and palometa (Pavlisko *et al.*, 1997). Chen *et al.* (2009) reported that pepsin 1 and 2 from snakehead were nearly completely inhibited by pepstatin with a pepstatin/pepsin molar ratio of 1:1, whereas pepsin 3 was not so sensitive: a 4-fold molar excess of pepstatin A was necessary for the complete inhibition. Pepsins 1 and 2 from North Pacific bluefin tuna were strongly inhibited by pepstatin whereas pepsin 3 was less sensitive to pepstatin. A 17-fold molar excess of pepstatin was required for complete inhibition of pepsin 3 (Tanji *et al.*, 1988). In this respect, pepsin 3 resembles mammalian pepsin C. Each tuna pepsin was less sensitive to diazoacetyl-DL-norleucine methyl ester than was porcine pepsin A. The effects of *p*-bromophenacyl bromide and 1,2-epoxy-3-(*p*-nitrophenoxy) propane varied among tuna pepsins (Tanji *et al.*, 1988).

The effects of some chemicals such as ATP and divalent cations on the activity of pepsin were demonstrated. ATP, $CaCl_2$, $MgCl_2$ and $CoCl_2$ affected the activities of pepsin A and B from pectoral rattail stomach differently (Klomklao *et al.*, 2007). ATP showed no impact on the activity of both pepsins activity even though the concentration increased. However, the activity of both pepsins A and B increased in the presence of divalent cations ($CaCl_2$, $MgCl_2$ and $CoCl_2$), especially when the concentration increased. Cathepsin D is activated by ATP when hemoglobin was used as a substrate (Pillai and Zull, 1985), whereas pepsins are not activated by ATP (Gildberg *et al.*, 1990; Pillai and Zull, 1985). Furthermore, the stimulating influence of divalent metallic ions revealed a difference between the pepsins and cathepsin D (Xu *et al.*, 1996). Gilderg *et al.* (1990)also found that ATP had no effect on the Atlantic cod pepsins, while Ca^{2+} and Cu^{2+} activated all pepsins. Xu *et al.* (1996) also reported that ATP did not alter the activity of orange roughy pepsins but divalent cations such as Ca^{2+} and Cu^{2+} slightly stimulated the activities.

1.2.2.4.6 Amino acid composition

The amino acid compositions of fish pepsins, in general, show similarities with porcine pepsin (Table 2). However, the relatively high content of basic amino acids gives an overall closer resemblance with the amino acid composition of porcine cathepsin D (Gildberg *et al.*, 1990). Pepsin I contains higher amounts of lysine, histidine and arginine, and lower amounts of aspartic acid and glutamic acid than Pepsin II. Therefore, pepsin I has much higher pI. Furthermore, the differences in glycosylation may also be of some importance (Gildberg *et al.*, 1990). The cystine content indicates three possible disulfide linkages per molecule of Pepsin I and four per molecule of Pepsin II (Gildberg *et al.*, 1990). Gildberg *et al.* (1990)concluded that the amino acid composition of the cod pepsins resembles porcine cathepsin D more than porcine pepsin. In addition, the glycoprotein nature of the cod pepsins reveals a molecular similarity with cathepsin D (Huang *et al.*, 1979). However, some mammalian pepsins are also glycosylated (Baudyš *et al.*, 1988). The amino acid composition of cod pepsins and sardine acid proteinases are fairly similar (Table 2). However, cod pepsins have serine content similar to that of porcine cathepsin D, which is significantly higher than the serine content of the sardine acid proteinases.

Table 2. Amino acid composition of Atlantic cod pepsins and sardine acid proteinases compared with porcine pepsin and cathepsin D

Amino acid	Number of amino acids in enzymes					
	Atlantic cod [(a)]		Sardine acid proteinase [(b)]		Porcine [(c)]	
	Pepsin I	Pepsin II[a]	I	II	Pepsin	Cathepsin D
Tryptophan	3	4	5	4	5	4
Lysine	13	11	7	6	1	18
Histidine	7	4	4	6	1	5
Arginine	12	6	3	6	2	11
Aspartic acid	25	30	40	33	42	29
Threonine	20	21	37	22	26	18
Serine	37	33	29	20	44	32
Glutamic acid	24	33	29	27	26	32
Proline	16	21	28	18	15	21
Glycine	36	38	34	36	35	31
Alanine	27	23	27	23	16	25
Cystine/2	6	8	9	7	6	8
Valine	27	28	22	26	22	23
Methionine	11	8	9	6	4	7
Isoleucine	19	18	14	14	26	15
Leucine	19	14	16	16	26	27
Tyrosine	15	10	9	11	16	13
Phenylalanine	15	13	8	10	14	10
Total	332	323	330	291	327	329

Sources: (a) Gildberg *et al*. (1990), (b) Noda and Murakami (1981) and (c) Cunningham and Tang (1976)

1.2.3 Aqueous two-phase systems

The downstream processing of enzymes and macromolecules generally requires purification techniques that preserve their biological activity. The conventional procedures include salt precipitation, chromatography, dialysis and filtration. These methods may result in a loss of biological activity with a concomitant poor yield of the final product (Spelzini *et al.*, 2006). The partitioning in aqueous two-phase systems (ATPS) is a good and selective method to be applied for biomolecule

purification, since such systems allow the removal of contaminants by a simple and economical process (Estela da Silva and Teixeira Franco, 2000; Spelzini *et al.*, 2005; Spelzini *et al.*, 2006). ATPS has a number of advantages with respect to the conventional methods for the isolation and purification of proteins: the partition equilibrium reaches very fast. ATPS can be applied in scale up and has the possibility of continuous steady state operation. Besides, its low cost and the materials that form this system are not expensive and can be recycled (Spelzini *et al.*, 2005). Aqueous two-phase partitioning is a potentially useful clarification or partial purification technique for a number of reasons: (1) it is a gentle method, having little or no adverse effect on the biological activity of most proteins; (2) many of the polymers used exhibit protein-stabilizing properties; (3) the yield of protein recovered is generally high and (4) little if any technical difficulties arise during process scale-up (Walsh, 2001).

1.2.3.1 Formation of aqueous two-phase system

Aqueous two-phase system (ATPS) is formed when two incompatible polymers or one polymer and an inorganic salt are mixed in water above certain critical concentrations (Oliveira *et al.*, 2002). Proteins are partitioned between two phases with a partition coefficient that can be modified by changing the medium experimental conditions such as pH, salts, ionic strength, etc (Spelzini *et al.*, 2005). Three types of ATPS (i.e. polymer–salt, polymer–polymer and others) have been traditionally used (Table 3). In the polymer–salt systems, polyethylene-glycol (PEG)–phosphate ATPS is commonly used, due to several advantages, including: low cost, wide past and current application and the range of system pH (from 6 to 9) under which the ATPS is stable (Rito-Palomares, 2004). In these extraction systems, the product of interest is concentrated in a phase that contains predominantly water and increased concentration of one of the phase forming components, in which the majority of the cases is PEG.

Table 3. General characteristics of selected ATPS processes to protein recovery

Type of ATPS	Biological origin	Target product	Product
Polymer–polymer			
PEG–dextran	*Aspergillus niger*	β-Glucosidase	95
PEG–starch	Wheat	α-Amylase	75
PEG–HPS	*Sccharomyces*	Alcohol	77–100
(EO–PO)–	Recombinant E. coli	Apolipo-protein	85–90
Polymer–salt			
PEG–	Bovine blood	BSA	85
	Brewers' yeast	Piruvate kinase	75
	Cheese whey	α-Lactoalbumin	65
	Aspergillus awamori	Glucoamylase	96
	Bovine brain	Prion proteins	N.r.
	Serum free	IgG	100
	Spirulina maxima	c-Phycocyanin	87
	Bakers' yeast	G3PDH	73
	E. coli	L1	65
	Bacillus pumilus	Alkaline xylanase	98
	Transgenic milk	Human al-	91
PEG–citrate	*E. coli*	Penicillin acylase	92
	Commercial source	Porcine insulin	N.r.
PEG–sulphate	Transgenic milk	Human al-	91

HPS, hydroxylpropyl starch; EO, ethylene oxide; PO, propylene oxide; N.r., not reported.

Source: Rito-Palomares (2004)

ATPS can be formed when two flexible chain polymers or one polymer and a salt are mixed; macromolecules are partitioned between the two phases and a partition coefficient (K) can be defined (Spelzini *et al.*, 2006) as follows:

$$K = \frac{[P]_T}{[P]_B}$$

where $[P]_T$ and $[P]_B$ are the macromolecule concentrations in the top and bottom phases, respectively.

The partition coefficients for proteins generally fall within the range of 0.1 to 10 (Gu, 2000). For large molecules (such as high molecular weight DNA and RNA) and particles (such as cells and viral particles), partition coefficients > 100 to < 0.01 are observed (Albertsson, 1986). Small ions tend to partition equally between the two phases (Gu, 2000). When the partition coefficients (or ratios) of two substances differ by a factor of 10 or more, their separation can be satisfactorily carried out (Reh *et al.*, 2002). The partition coefficient of a protein depends on different factors such as the molecular mass of the polymer and of the macromolecule, pH, the presence of salts and ions in the medium, macromolecular hydrophobicity, etc (Spelzini *et al.*, 2006).

1.2.3.2 Factors affecting aqueous two-phase systems and partitioning

1.2.3.2.1 Effect of polymer, molecular weight and concentration

Molecular weight of the polymers strongly affects the partitioning of a protein in PEG-dextran-water systems (Albertsson, 1958). Kula (1979) proposed that if it is desirable to have a higher partition coefficient, lowering the average PEG molecular weight may help. The effect of polymer molecular weight depends on molecular weight of the solute (Albertsson, 1986). Polymers with higher molecular weights and higher concentrations usually bring higher viscosities to the liquid solutions (Gu, 2000). The MW and concentration of PEG were found to have significant effects on protein partition. The extraction with low MW PEG4000 showed the best conditions for the partitioning of tannery wastewater protein in PEG + $MgSO_4$ + water system (Saravanan *et al.*, 2007). Spelzini *et al.* (2006) found that the increase of PEG concentration for partitioning of chymosin and pepsin favored the protein transfer to the top phase. Klomklao *et al.* (2005) revealed that phase compositions including PEG molecular mass and concentration as well as types and concentrations of salt affected the partitioning of proteinase from the spleen of yellowfin tuna (*Thunnas albacores*).

1.2.3.2.2 Effect of temperature

Temperature affects the phase separation. When temperature is decreased, phase separation occurs at lower polymer concentrations for PEG-dextran-

water systems. Thus PEG and dextran are needed to achieve phase separation (Gu, 2000). The opposite is true for PEG-salt-water systems (Albertsson and Tjerneld, 1994). Temperature also changes the partitioning of biomolecules. Partition coefficients of lysozyme and catalase and their ratios in different PEG-dextran-water systems were observed at different temperatures (Zaslavsky, 1994). A lower temperature tends to provide a better separation of the two proteins. Kula (1979) also pointed out that K values are usually higher at a lower temperature. Using a lower temperature will cause the viscosity to be higher. Room temperature can be used with minimal bioactivity losses. Therefore, chilling aqueous two phase systems is usually not required, unless very fragile proteins are involved (Kula, 1979).

1.2.3.2.3 Effect of Salt

Salts at moderate concentrations have the effects on the phase separation of nonionic polymer-polymer-water systems (Gu, 2000). Usually a much lower polymer concentration is required for phase separation when the salt concentration increases (Albertsson and Tjerneld, 1994). Salt can be used rather effectively to change the partition coefficient of biomolecules. At low salt concentrations (0.1 to 0.2 M), the effects of salt type and concentration can be dramatic for proteins at pH far away from their isoelectric points (Diamond and Hsu, 1992). As a rule of thumb, the decrease of partition coefficient for negatively charged proteins in PEG-dextran-water systems is sulfate > fluoride > acetate > chloride > bromide > iodide and lithium > ammonium > sodium > potassium. Positively charged proteins follow the opposite trend (Diamond and Hsu, 1992). Albertsson (1986) reported that increasing NaCl concentration in the range of 0 to 5 M greatly increased the partition coefficient of several proteins (phycocyanin, phycoerythrin, gamma globulin, ceruloplasmin, and serum albumin) in a phase system containing 4.4% PEG 8000 and 7% dextran at pH 6.8.

1.2.3.3 Partitioning and recovery of proteinases by ATPS

ATPS has been used successfully for partitioning and recovery some proteinases. Klomklao *et al.* (2005) demonstrated the partitioning of spleen proteinase from yellowfin tuna (*Thunnus albacores*) using an ATPS and found that phase

compositions including PEG molecular mass and concentration as well as types and concentration of salts affected protein partitioning. ATPS comprising PEG1000 (15%, w/w) and magnesium sulfate (20%, w/w) provided the best condition for the maximum partitioning of the proteinase into the top phase and gave a highest specific activity (47.0 units/mg protein) and purification fold (6.61). The yield of 69.0% was obtained. Under the same ATPS condition used, the partitioning of proteinase of splenic extract from three tuna species involving skipjack tuna, yellowfin tuna and tongol tuna were compared. The purity of splenic extract from all tuna species increased after ATPS process. Among all species tested, yellowfin tuna showed the highest purification fold, followed by tongol tuna and skipjack tuna, respectively. SDS-substrate gel electrophoresis revealed that the band intensity of major proteinase in ATPS fraction from all tuna species slightly increased with the concomitant decrease in band intensity of other contaminating proteins, indicating the greater specific activity of splenic extract. The partition behavior of trypsin in poly(ethylene glycol) (PEG)--cashew-nut tree gum aqueous two-phase systems was studied by Oliveira *et al*. (2002). The system properties had little effect on trypsin partition coefficients. In some cases, the NaCl addition changed dramatically the partition coefficient. Altering the conditions allows the manipulation of the protein partition (Oliveira *et al.*, 2002). The maximum recovery of trypsin activity in the cashew-nut tree gum phase was obtained with ATPS containing PEG (molecular weight 8000) and 1.0 M NaCl at pH 7.0. The partitioning of bovine trypsin and α-chymotrypsin, proteases of similar physico-chemical properties, in different PEG/sodium citrate ATPS has also been investigated (Tubío *et al.*, 2007). Both a decrease in PEG molecular weight and an increase in pH led to a higher partition coefficient for both enzymes. Tubío *et al.* (2007) revealed that ATPS formed by PEG of molecular weight 3350 and citrate pH 5.2 shows the best separation capability which was enhanced in the presence of 3% sodium chloride. The transfer of both proteins to the top phase was associated with negative enthalpic and entropic changes.

The partitioning of chymosin (from *Aspergilus niger*) and pepsin (from bovine stomach) was carried out using ATPS containing PEG-potassium phosphate. Spelzini *et al.* (2005) found that the partition performed at pH 7.0 showed high affinity of both enzymes for the PEG rich phase. The increase of PEG concentration

favored the protein transfer to the top phase, suggesting an important protein–polymer interaction. PEG shows a stabilizing effect on the chymosin and pepsin (Spelzini *et al.*, 2005). The influence of the phase volume ratio and polymer pausidispersity on chymosin and pepsin partition in polyethylenglycol–phosphate ATPS was also studied (Spelzini *et al.*, 2006). Both proteins showed a high affinity for the PEG rich phase with a partition coefficient from 20 to 100 for chymosin and from 20 to 180 for pepsin, when the PEG molecular mass in the system varied between 1450 and 8000. The partition coefficient of chymosin was not affected by the volume phase ratio, while a significant decrease in the partition coefficient of pepsin was found with the increase in the top/bottom phase volume ratio (Spelzini *et al.*, 2006). Pepsin was found to be partitioned towards the PEG-rich phase, suggesting an important protein–polymer interaction due to the highly hydrophobic character of the protein surface exposed to the solvent (Imelio *et al.*, 2008). The combination of both partition in PEG 1450-phosphate system and chitosan precipitation has been used to purify pepsin from bovine abomasum homogenate (Boeris *et al.*, 2009). Pepsin recovery of 48.5% with a purification factor of 9.0 was obtained and biological activity of the recovered enzyme remained unaltered. The interaction between pepsin and non-charged PEG polymer was studied. Spelzini *et al.* (2008) found that PEG1450 had a higher interaction capacity with the protein than PEG8000. PEG1450 showed interpolymer interaction, leading to the complex formation, whereas PEG8000 showed a cooperative interaction between the polymer and protein molecules which was independent of the PEG concentration.

1.2.4 Applications of pepsin

Pepsin can be used for a number of applications, mainly for hydrolysis proposes. Maximization of pepsin could enlarge the efficacy of enzyme. Due to the specificity, pepsin could be used to cleave peptides specifically for collagen extraction.

1.2.4.1 Cheese production

Pepsin from Atlantic cod (*Gadus morhua*) can be used as a coagulating enzyme for the preparation of Cheddar cheese. When aged less than 6

months, the cheese was judged acceptable by sensory panels. However, this enzyme resulted in high loss of protein and fat to the whey during cheese making and the product developed bitterness and pasty consistency after prolonged aging (Brewer *et al.*, 1984). Cod pepsin, like porcine pepsin, has a low ratio of milk clotting activity at pH 6.2 (clot units, CU) to hemoglobin hydrolysis activity at pH 1.9 (pepsin units, PU) compared to calf chymosin and is not stable above pH 6.4 in milk substrate (Brewer *et al.*, 1984). These properties are undesirable for certain types of cheese making. A catalyst having high molecular activity at low reaction temperature and low thermal stability, i.e. which can be readily inactivated after completion of the desired reaction, may be advantageous in certain industrial applications (Simpson and Haard, 1984). Since cod pepsin has some properties which are not desirable for conventional cheese making, though it has a relatively low temperature coefficient and low temperature optimum for protein hydrolysis. Haard (1986) explored the properties of this enzyme with casein and milk substrate with an aim of exploiting it as an advantageous catalyst for carrying out the enzymic phase of milk clotting at low temperatures. The temperature coefficient for the enzymic phase of milk clotting was lower for cod gastric protease than for calf rennet and various other rennet substitutes. Immobilization of cod pepsin on Sepharose resulted in an increase in Arrhenius activation energy for hemoglobin hydrolysis from 8.5 Kcal/mole to 12.8 Kcal/mole. Sepharose-cod protease did not catalyze the enzymic phase of milk clotting. Cold renneting of milk substrate with cod gastric protease at 0°C resulted in continued formation of nonprotein nitrogen (NPN) after the enzymic phase of milk clotting was complete. Initiation of milk clotting by raising the temperature to 39°C prevented the subsequent formation of NPN. Pepsin A isolated from the gastric mucosa of two year old harp seal (*Pagophilus groenlandicus*) has also been studied for cheese making. Shamsuzzaman and Haard (1985) reported that seal pepsin A has a relatively high CU/PU ratio of 0.074, although it is lower than that of calf chymosin (0.170). Equivalent milk clotting units of chymosin and seal pepsin A catalyzed the formation of the same amount of NPN when incubated with 2% casein and for both there was no appreciable increase in NPN formation in the second hour of incubation. Seal pepsin A was similar to chymosin in clotting milk substrate with respect to the influence of pH, dilution, calcium chloride and

temperature. Cheddar cheeses prepared with either calf rennet or pepsin A as coagulating agent were similar in yield, chemical composition and taste as judged by preference tests.

1.2.4.2 Collagen and gelatin preparation

Pepsin has been largely used to solubilize collagens from several tissues. In certain conditions, pepsin can extract relatively intact triple-helical collagenous molecules. Up to 80% to 90% (depending on the source) of native skin collagens can be extracted by limited pepsin digestion using a defined enzyme to substrate ratio and low temperature (Chomarat *et al.*, 1994). Generally, use of pepsin in combination with acid extraction increased the yield of collagen. Nagai *et al*. (2002) extracted and characterized pepsin-solubilized collagen (PSC) from ocellate puffer fish skin. It was a heterotrimer with a chain composition of $(\alpha1)_2\alpha2$. The denaturation temperature was 28 °C, about 9 °C lower than that of porcine skin collagen. The yield of PSC was higher (44.7%) than acid-solubilized collagen (10.7%). Nagai *et al.* (2000) found that 35.2% collagen was extracted from rhizostomous jellyfish (*Rhopilema asamushi*) by limited pepsin digestion. The primary structure was very similar to that of pepsin-solubilized collagen from edible jellyfish mesogloea, but it was different from those of the collagen from edible jellyfish exumbrella and the acid-soluble collagen from its mesogloea. The rhizostomous jellyfish mesogloea collagen had a denaturation temperature (T_d) of 28.8 °C. This collagen contained a large amount of a fourth subunit, designated as α4. This collagen may have the chain composition of an α1α2α3α4 heterotetramer. Nagai and Suzuki (2002) found that the collagen extracted from the outer skin of the paper nautilus was hardly solubilized in 0.5 M acetic acid. The insoluble matter was easily digested by 10% pepsin (w/v), and a large amount of collagen was obtained with about a 50% yield (pepsin-solubilized collagen). The PSC had a chain composition of α1 α2 α3 heterotrimer similar to *Callistoctopus arakawai* arm collagen. Furthermore, collagen from the outer skin of cuttlefish (*Sepia lycidas*) was extracted by Nagai *et al*. (2001). The initial extraction of the cuttlefish outer skin in acetic acid yielded only 2% of collagen (dry weight basis). On subsequent digestion of the residue with 10% pepsin (w/v), pepsin-solubilized collagen (PSC) was obtained with a yield of 35% (dry weight basis). PSC was extracted

from the skin of grass carp (*Ctenopharyngodon idella*) with the yield of 46.6%, on a dry weight basis (Zhang *et al.*, 2007). Extraction and some properties of PSC from the skin of bigeye snapper (*Priacanthus tayenus*) were also investigated. Nalinanon *et al.* (2007) found that addition of bigeye snapper pepsin (BSP) at a level of 20 kUnits/g of defatted skin resulted in an increased content of collagen extracted from bigeye snapper skin. The yields of collagen from bigeye snapper skin extracted for 48 h with acid and with BSP were 5.31% and 18.74% (dry basis), respectively. With pre-swelling in acid for 24 h, collagen extracted with BSP at a level of 20 kUnits/g of defatted skin for 48 h had a yield of 19.79%, which was greater than that of collagen extracted using porcine pepsin at the same level (13.03%). Chomarat *et al*. (1994) compared the effectiveness of two proteolytic enzyme; pepsin and proctase (isolated from *Aspergillus niger*) for the solubilization of collagen from bovine skin. Some of the molecular properties of gelatins derived from these collagens were also studied. Pepsin and proctase solubilized collagens with the similar yields (75% and 76% of total collagen as calculated from hydroxyproline). Recently, collagen from the skin of brownbanded bamboo shark (*Chiloscyllium punctatum*) was isolated by Kittiphattanabawon *et al.* (2010). The use of porcine pepsin at level of 20 units/g residual skin after acid extraction increased the yield by 8.86% (wet wt.). Pepsin hydrolysis had no effect on triple-helical structure of collagen as determined by FTIR.

Recently, Nalinanon *et al.* (2008a) developed the process for gelatin extraction from bigeye snapper (*Priacanthus tayenus*) skin by using pepsin as the aid in combination with a protease inhibitor. The extraction efficiency was augmented by an acid-swelling process in the presence of bigeye snapper pepsin (BSP) at a level of 15 units/g alkaline treated skin at 4°C for 48 h followed by extraction at 45°C for 12 h with a yield of 40.3% (based on hydroxyproline content). Incorporation of soybean trypsin inhibitor at a concentration of 0.1 μM retarded the degradation of resulting gelatin caused by endogenous serine proteinases (Nalinanon *et al.*, 2008a).

1.2.4.3 Production of protein hydrolysate

Uses of pepsin for production of protein hydrolysate have been widely developed. A high quality white, fluffy, water-soluble fish protein hydrolysate (FPH) powder can be prepared by pepsin hydrolysis of fish flesh (Liu and Pigott, 1981). Liu

and Pigott (1981) reported that the digestion temperature should be increased to about 62°C with continuous stirring for 4 hr, and concentration of crude pepsin can be about 6-9% since the activity of 6% crude pepsin showed an insignificant difference from that 9% crude pepsin. Surówka and Fik (1994) recovered proteinaceous substances from chicken heads with the aid of pepsin. Minced heads of broiler chickens were hydrolyzed at 55°C and pH 1.5 in the presence of 750 g water and 3 g pepsin per kg raw material. After 5 h of proteolysis, 1 kg of the raw material yielded 144 g of dry unneutralized hydrolysate containing 15.7 g total nitrogen, equivalent to a 67.8% nitrogen recovery. Neutralization prior to drying marginally decreased nitrogen recovery and reduced the nitrogen solubility index by > 30%. The final dried products were pale cream colored, had no bitter taste, were of high microbiological quality and had a high mineral content. However, both dried hydrolysates had poor emulsifying properties (Surówka and Fik, 1994). Pepsin has also been used to hydrolyze Hoki frame protein, normally discarded from industrial fish plants. High antioxidative activity was observed in the resulting hydrolysates (Je *et al.*, 2005). Hoki frame protein hydrolysates (HPH) prepared by pepsin were fractionated according to the molecular mass into four fractions types, HPH I (5–10 kDa), HPH II (3–5 kDa), HPH III (1–3 kDa), and HPH IV (below 1 kDa), using an ultrafiltration membrane. HPH III showed a higher antioxidative activity than other hydrolysates in a linoleic acid emulsion system. In addition, the free radical scavenging activities of the fractionated hydroysates were evaluated using electron spin resonance spectroscopy. The results showed that HPH III has the highest scavenging effects towards 1,1-diphenyl-2-picrylhydrazyl, hydroxyl, alkyl and superoxide anion radicals, and the inhibition efficacy was dose-dependent (Je *et al.*, 2005). Additionally, hoki (*Johnius belengerii*) frames with flesh and skeleton discarded from industrial processing were degraded by pepsin in acetic acid solution (pH 2.2) (Jung and Kim, 2007). After digestion, a calcium-binding peptide was isolated from the pepsinolytic hydrolysates using a hydroxyapatite affinity chromatography and was identified as Val-Leu-Ser-Gly-Gly-Thr-Thr-Met-Tyr-Ala-Ser-Leu-Tyr-Ala-Glu (MW: 1,561 Da) (Jung and Kim, 2007). Furthermore, production of protein hydrolysate from plants has also been investigated. Bejosano and Corke (1999) demonstrated the feasibility to produce protein hydrolysates from protein concentrates, by-product of *Amaranthus* and

buckwheat starch extraction. Protein hydrolysate was prepared by adjusting the protein concentration to 1% (w/v) in 0.01 N HCl (pH 2.0) and then adding 91 units of pepsin A (Sigma, St Louis, MO, USA) per mg solid to the suspension at the rate of 0.2 mg ml^{-1}. The mixture was incubated at 37°C with mild shaking for 16 h, after which pH was adjusted to 7.5 and kept at 5°C for 72 h. The pH was then adjusted to 6.5, followed by freeze-drying.

Pepsinolytic oligopeptide-enriched hydrolysate from globin, derived from porcine hemoglobin, with angiotensin-I-converting enzyme (ACE) inhibitory activity was prepared (Yu *et al.*, 2006). Globin solution was digested by pepsin (porcine stomach mucosa) with a ratio of protein substrate to enzyme (100:1, w/w) at pH 2.0 and a temperature of 37°C for 12 h, and then heated at 100°C for 10 min to inactive the pepsin. The hydrolysate was then separated on Sephadex LH-20 gel filtration column and five major fractions (I, II, III, IV and V) were obtained. Biological functions of the hydrolysate and five fractions were assayed. Hydrolysate and fractions IV and V had good ACE inhibitory activity, with IC_{50} values of 1.19, 0.67, 0.10 mg/ml, respectively. Tomita *et al*. (1991) prepared the hydrolysates by cleavage of lactoferrin with porcine pepsin, cod pepsin, or acid protease from *Penicillium duponti*. Those hydrolysates showed strong inhibitory activity against *Escherichia coli* O111, whereas hydrolysates produced by trypsin, papain, or other neutral proteases were much less active. Low molecular weight peptides generated by porcine pepsin cleavage of lactoferrin showed broad-spectrum antibacterial activity, inhibiting the growth of a number of Gram-negative and Gram-positive species, including strains that were resistant to native lactoferrin (Tomita *et al.*, 1991). The inhibitory effect of a pepsin hydrolysate of bovine lactoferrin on ACE has been examined by Ruiz-Giménez *et al.* (2007). *In vitro* assays showed that lactoferrin hydrolysate had inhibitory effect on ACE activity with an IC_{50} value of 0.95±0.06 mg mL^{-1}, which possessed a potential to modulate hypertension, Recently, Lee *et al.* (2010) hydrolyzed tuna frame protein using Alcalase, Neutrase, pepsin, papain, α-chymotrypsin and trypsin. Among those enzymes used, peptic hydrolysate exhibited the highest ACE inhibitory activity. A potent ACE inhibitory peptide from tuna frame protein was identified as Gly-Asp-Leu-Gly-Lys-Thr-Thr-Thr-Val-Ser-Asn-Trp-Ser-Pro-Pro-Lys-Try-Lys-Asp-Thr-Pro (MW:2,482 Da, IC50:11.28 μM).

This peptide was a non-competitive inhibitor against ACE (Lee *et al.*, 2010). Furthermore, the oyster protein hydrolysate prepared by pepsin treatment exhibited antihypertensive activity when it was orally administered to spontaneously hypertensive rat at a dose of 20 mg/kg (Wang *et al.*, 2008). A purified peptide, Val-Val-Tyr-Pro-Trp-Thr-Gln-Arg-Phe, from oyster protein hydrolysate digested with pepsin also exhibited ACE inhibitory activity with IC_{50} value of 66 μmol/L *in vitro* (Wang *et al.*, 2008).

1.3 Objectives

1. To purify and characterize pepsinogen and pepsin from stomach of different tuna species.
2. To investigate the use of aqueous two-phase systems (ATPS) for partitioning and recovery of pepsin from tuna stomach.
3. To study the use of pepsins from the stomach of different tuna species for collagen extraction from the skin of different fish species.
4. To elucidate the hydrolysis mechanism of type I collagen by tuna pepsins and porcine pepsin.
5. To study the functionalities and antioxidative properties of the protein hydrolysate prepared from fish muscle using tuna pepsin.

CHAPTER 2

BIOCHEMICAL PROPERTIES OF PEPSINOGEN AND PEPSIN FROM THE STOMACH OF ALBACORE TUNA (*THUNNUS ALALUNGA*)

2.1 Abstract

Pepsinogen (PG) from the stomach of albacore tuna (*Thunnus alalunga*) was purified to homogeneity by using a series of chromatographies involving Sephacryl S-200HR, Sephadex G-50 and DEAE-cellulose with a 658-fold increase in purity. Based on the native-PAGE and zymography, PG showed a single band with pepsin activity. Molecular weights (MW) of PG and active pepsin were estimated to be 39.9 and 32.7 kDa as determined by SDS-PAGE, respectively. PG was converted to the corresponding pepsin through an intermediate form (MW≈36.8 kDa) and the complete activation was observed after 30-60 min. The N-terminal amino acid sequence of the first 15 amino acids of activation segment of pepsinogen was FHKLPLIKGKTAREE. The optimal pH and temperature for pepsin activity were 2.0 and 50 °C, respectively. The activity was stable in the pH range of 2-5. Residual activity more than 85% was found after heating at temperatures up to 50 °C for 30 min. Pepsin activity was strongly inhibited by pepstatin A, while E-64, ethylenediaminetetraacetic acid (EDTA) and soybean trypsin inhibitor exhibited the negligible effect. SDS and cysteine also showed inhibitory effects, while ATP, molybdate, NaCl and $CaCl_2$ had no impact on pepsin activity.

2.2 Introduction

Pepsin is the major digestive enzyme in stomach of animals, which is secreted as pepsinogen from chief cells of oxyntic glands located in the stomach wall epithelium (Kageyama, 2002). In acidic environment, pepsinogen 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 (Richter *et al.*, 1998). Pepsin and pepsinogen from stomach of various fish species such as polar cod (Arunchalam and Haard, 1985), North Pacific bluefin tuna (Tanji *et al.*, 1988), Atlantic cod (Gildberg *et al.*, 1990), sea bream (Zhou *et al.*, 2007), African coelacanth (Tanji *et al.*, 2007), pectoral rattail (Klomklao *et al.*, 2007), smooth hound (Bougatef *et al.*, 2008), Mandarin fish (Zhou *et al.*, 2008) and European eel (Wu *et al.*, 2009a) have been purified and characterized.

Albacore tuna (*Thunnus alalunga*), also known as longfin tuna, is recognized by its remarkably long and slender pectoral fins, about 30% of its fork length (Wu *et al.*, 2009b). It has a high commercial value and has served as an important raw material for the production of canned tuna in Thailand. During canned tuna manufacturing, a high amount of viscera containing various proteases is generated. Stomach proteases, especially pepsin, can be recovered and used, mainly for hydrolysis purposes. Fish pepsin has been used for preparation of protein hydrolysate (Pavlisko *et al.*, 1997) and for extraction of collagen (Nalinanon *et al.*, 2007) and gelatin (Nalinanon *et al.*, 2008a). Recently, stomach extract from albacore tuna has been used as the potential aid for collagen extraction from the skin of threadfin bream (Nalinanon *et al.*, 2008b). Therefore, albacore stomach can be used as a promising source of pepsin for further applications. However, no information regarding the biochemical characteristics of pepsinogen and pepsin from the stomach of albacore tuna has been reported. Therefore, the aims of this study were to purify pepsinogen from albacore tuna stomach and to characterize some biochemical properties of the corresponding pepsin. For pepsin from tongol tuna, it was not purified and characterized due to the less abundance for tuna processing in Thailand.

2.3 Materials and Methods

2.3.1 Chemicals

Bovine hemoglobin, *β*-mercaptoethanol (βME), *L*-cysteine, adenosine 5′-triphosphate (ATP), molybdate, pepstatin A, ethylenediaminetetraacetic acid (EDTA), soybean trypsin inhibitor, 1-(L-trans-epoxysuccinyl-leucylamino)-4-guanidinobutane (E-64) and bovine serum albumin were purchased from Sigma Chemical Co. (St. Louis, MO, USA). Folin-Ciocalteu's phenol reagent was obtained from Merck (Darmstadt, Germany). Sephacryl S-200HR and Sephadex G-50 were procured from Pharmacia Biotech (Uppsala, Sweden). Diethylaminoethyl (DEAE)-cellulose was obtained from Whatman (Maidstone, England). Sodium dodecyl sulfate (SDS), Coomassie Blue R-250, and *N,N,N′,N′*-tetramethylethylene diamine (TEMED) were procured from Bio-Rad Laboratories (Hercules, CA, USA).

2.3.2 Preparation of fish stomach

Frozen albacore tuna (*T. alalunga*), off-loaded approximately a month after capture and freezing, were obtained from Tropical Canning (Thailand) Public Co., Ltd., Songkhla, Thailand. After thawing, the internal organs from 50 fish were collected and packed in polyethylene bag, kept in ice with a solid/ice ratio of 1:2 (w/v) and transported to the Department of Food Technology, Prince of Songkla University, Hat Yai within 1 h. Upon the arrival, internal organs were excised and only stomach was collected and placed in polyethylene bag. The collected stomach was stored at -20 °C until use.

2.3.3 Preparation of crude extract

Frozen stomach was thawed using running water (26-28 °C) until the core temperature reached -2 to 0 °C. The sample was cut into pieces with a dimension of 0.5 × 0.5 cm^2. Sample was finely ground in liquid nitrogen using a National Model

MX-T2GN blender (Taipei, Taiwan). Sample was then lyophilized using an EYELA FDU-1200 freeze-dryer (Tokyo Rikakikai Co., Ltd., Tokyo, Japan) and the dry powder was referred to as 'stomach powder'.

To prepare the crude extract, stomach powder was suspended in 20 mM sodium phosphate buffer (pH 7.0) containing 1 mM phenylmethylsulfonyl fluoride PMSF at a ratio of 1:9 (w/v) and stirred continuously at 4 °C for 3 h. The suspension was centrifuged for 20 min at 4 °C at 20,000 × *g* (H-2000B, Kokusan, Tokyo, Japan) to remove the tissue debris. The supernatant was collected and lyophilized. Before used, the dry extract powder (10 g) was dissolved with 60 mL of 20 mM sodium phosphate buffer (pH 7.0) referred to as 'starting buffer; SB', followed by centrifugation at 4 °C for 10 min at 10,000 × *g*. The supernatant was collected and referred to as "crude extract".

2.3.4 Purification of albacore tuna pepsinogen

All steps of purification were carried out at 4 °C. Crude extract was applied onto Sephacryl S-200HR column (3.9 × 70 cm), previously equilibrated with approximately two bed volumes of SB. Elution was carried out using the same buffer at a flow rate of 0.5 mL/min. Fractions of 4.5 mL were collected and those with proteolytic activity were pooled and lyophilized. Lyophilized Sephacryl S-200HR fraction was dissolved in SB prior to loading onto Sephadex G-50 (Pharmacia Biotech, Uppsala, Sweden) (3.9 × 70 cm) column equilibrated with approximately two bed volumes of SB. The proteases were eluted with SB at the flow rate of 0.5 mL/min and fractions of 4.5 mL were collected. Those fractions with proteolytic activity were pooled and lyophilized. Lyophilized Sephadex G-50 fraction was dissolved in distilled water and dialyzed against 60 volumes of SB for 12 h. The dialysate was then subjected to DEAE-cellulose (Whatman, Maidstone, England) column (2.2 × 35 cm) equilibrated with SB. After the column was washed with SB until A_{280} was below 0.05, the elution was performed with a linear gradient of 0-0.75 M NaCl in SB at a flow rate of 0.5 mL/min. Fractions of 4.5 ml, with proteolytic activity were pooled and dialyzed against 60 volumes of SB for 12 h prior to lyophilization. The lyophilized fraction was dissolved in SB and re-chromatographed

onto Sephadex G-50 column, followed by the elution with SB at the flow rate of 0.5 mL/min. Fractions (2.5 mL) were collected and those with proteolytic activity were pooled and lyophilized. The lyophilized pooled fraction was dissolved and dialyzed prior to loading onto DEAE-cellulose column (2.2 × 35 cm) in the same manner described previously. The elution was performed with a linear gradient of 0.2-0.4 M NaCl in SB at a flow rate of 0.25 mL/min. Fractions (2 mL) were collected and those with proteolytic activity were combined and dialyzed against 60 volumes of SB for 12 h. Dialysate obtained was lyophilized.

During purification, protein concentration was measured by monitoring the absorbance at 280 nm, and the proteolytic activity was measured using hemoglobin as a substrate at pH 2.0 and 50 °C as per the method of Nalinanon *et al.* (2008b).

2.3.5 Protein determination

Protein concentration was determined by measuring the absorbance at 280 nm or by the method of Lowry *et al.* (1951) using bovine serum albumin as a standard.

2.3.6 Assay of proteolytic activity

Proteolytic activity of purified enzyme was determined using hemoglobin as a substrate according to the method of Nalinanon *et al.* (2008b) with some modifications. To initiate the reaction, 200 μL of enzyme solution were added into the assay mixture containing 200 μL of 2% hemoglobin, 200 μL of distilled water and 625 μL of reaction buffer. Appropriate dilution was made to ensure that the amount of enzyme was not excessive for available substrate in the assay system. The reaction was conducted at pH 2.0 and 50 °C for 20 min. To terminate enzymatic reaction, 200 μL of 50% (w/v) trichloroacetic acid (TCA) were added. Unhydrolyzed protein substrate was allowed to precipitate for 1 h at 4 °C, followed by centrifuging at 15,000 × *g* for 10 min using a KUBOTA 3630 centrifuge (SiGMA

Laborzentrifugen, Osterode am Harz, Germany). The oligopeptide content in the supernatant was measured at 280 nm. One unit of activity was defined as the amount causing an increase of 1.0 in absorbance at 280 nm per min. A blank was run in the same manner, except that the enzyme was added into the reaction mixture after the addition of 50% (w/v) TCA.

2.3.7 Polyacrylamide gel electrophoresis (PAGE)

SDS-PAGE was performed according to the method of Laemmli (1970). Protein solutions were mixed at 1:1 (v/v) ratio with the SDS-PAGE sample buffer (0.125 M Tris-HCl, pH 6.8; 4% SDS; 20% glycerol; 10% βME) and boiled for 3 min. The samples (15 μg) were loaded onto the gel made of 4% stacking and 12.5% separating gels and subjected to electrophoresis at a constant current of 15 mA/gel using a AE-6440 electrophoresis apparatus (Atto Co., Tokyo, Japan). After electrophoresis, the gels were stained with 0.1% Coomassie brilliant blue R-250 in 50% methanol and 7% acetic acid and destained with 7% acetic acid.

Native-PAGE was performed using 12.5% separating gel in a similar manner with SDS-PAGE, except that the sample was not heated and SDS and reducing agent were left out.

2.3.8 Enzyme zymography

Zymography of purified pepsinogen (PG) from the stomach of albacore tuna was performed as per the method of Díaz-López *et al.* (1998) and Nalinanon *et al.* (2008b) with a slight modification. Enzyme solution was applied onto native-PAGE in the same manner as described previously. Electrophoresis was performed at constant current of 15 mA/gel at 4 °C. After electrophoresis, gels were removed and soaked in 0.1 M HCl to reduce the pH to 2.0 to activate the pepsin. After 15 min, the gels were soaked in hemoglobin solution (0.25% hemoglobin in 0.1 M glycine-HCl, pH 2.0) with constant agitation for 30 min at 4 °C to allow the substrate to penetrate into the gels. The gels were then soaked for 90 min in a fresh hemoglobin solution at 50 °C to develop the activity zone. After development of activity zone,

gels were washed using distilled water and fixed for 15 min in 12% TCA solution prior to staining and destaining as described previously. Development of clear zones on blue background indicated proteolytic activity.

2.3.9 Analysis of amino acid sequence

To analyze the N-terminal sequence, the PG was electroblotted onto polyvinylidenedifluoride (PVDF) membrane (ProBlott®, Applied Biosystems, Foster City, CA, USA) after SDS–PAGE. The amino acid sequence of the enzyme was analyzed using a protein sequencer, Procise 492 (Perkin Elmer, Foster City, CA, USA).

2.3.10 Biochemical properties

2.3.10.1 Pepsinogen activation

The conversion reaction from pepsinogen (PG) to pepsin was monitored at various times. PG solution (25 µL) was mixed with 25 µL of 0.5 M glycine-HCl (pH 2.0). The mixture was incubated at 4 °C for different times (0, 0.25, 0.50, 1, 5, 10, 30 and 60 min). At the time designated, 50 µL of SDS-PAGE sample buffer were immediately added to terminate the conversion reaction. The reaction mixtures were then analyzed by SDS-PAGE as previously described.

2.3.10.2 pH and temperature profile

To study pH profile, proteolytic activity of purified enzyme was measured at 50 °C using hemoglobin as a substrate at different pHs (pHs 1.0, 1.5 and 2.0 using 20 mM maleate buffer and pHs 2.0, 2.5, 3.0, 3.5, 4.0, 5.0, 6.0 and 7.0 using McIlvaine's buffer (0.2 M Na-phosphate and 0.1 M Na-citrate)). For temperature profile, the assay was performed at various temperatures (20, 30, 40, 45, 50, 55, 60 and 70 °C) at pH 2.0.

2.3.10.3 pH and thermal stability

For pH stability study, purified enzyme solution was incubated at pHs 2.0, 3.0, 4.0, 5.0, 6.0 and 7.0 using McIlvaine's buffer at room temperature for 30 min. Residual activity was then determined. For thermal stability, the purified enzyme solution was incubated at different temperatures (20, 30, 40, 50, 60 and 70 °C) for 30 min before rapid cooling using iced water. The residual activity was then assayed using hemoglobin as a substrate at pH 2.0 and 50 °C for 20 min.

2.3.10.4 Effect of some protease inhibitors

Purified enzyme solution (200 μL) was mixed with 200 μL of single protease inhibitor to obtain the final designated concentration (0.1, 1 and 10 μM for pepstatin A, 0.1 mM for E-64, 0.1 mM for soybean trypsin inhibitor and 2 mM for EDTA). Mixtures were incubated at room temperature for 30 min. The residual activity was measured at pH 2.0 and 50 °C. Relative activity was then calculated.

2.3.10.5 Effect of some chemicals

Different chemicals (200 μL) were mixed with the enzyme solution (200 μL) to obtain the concentration designated (0.5 mM for ATP and molybdate; 5 mM for NaCl and $CaCl_2$; 0.05 % (w/v) for SDS and 50 mM for *L*-cysteine). The mixtures were kept at room temperature for 30 min. The remaining activity was determined at pH 2 and 50 °C and the relative activity was calculated.

2.3.11 Statistical analysis

All experiments were run in triplicate. Data were subjected to Analysis of Variance (ANOVA) and mean comparisons were carried out using Duncan's multiple range test. SPSS statistic program (SPSS 11.0 for Windows, SPSS Inc., Chicago, IL, USA) was used for data analysis.

2.4 Results and Discussion

2.4.1 Purification of pepsinogen

Purification of pepsinogen from the stomach of albacore tuna is summarized in Table 4. The purification of pepsinogen was started by separating the crude extract using Sephacryl S-200HR column. After separation, the fractions with pronounced proteolytic activity were pooled and subjected to further purification. Purity of 2-fold was observed in Sephacryl S-200HR fraction. A single activity peak was obtained for this fraction upon the subsequent chromatography on Sephadex G-50 (data not shown). The yield of 25% and the purity of 168-fold were observed after gel filtration using Sephadex G-50 column. The pooled activity fractions were then subjected to ion-exchange chromatography using DEAE-cellulose. Ion-exchange chromatography was used to remove contaminating proteins and to separate different pepsinogen and pepsin isoforms (Klomklao et al., 2007; Tanji et al., 1988; Zhou et al., 2007). Pooled fractions from single activity peak obtained after DEAE-cellulose chromatography showed high purity of 513-fold with 17% yield. The results showed that Sephadex G-50 and DEAE-cellulose chromatographies effectively removed the contaminants from the crude extract. However, some protein contaminants were still retained as examined by PAGE (data not shown). In order to completely remove those contaminants, the selected fractions were re-chromatographed onto Sephadex G-50 and DEAE-cellulose columns, respectively. After the final purification step, purification fold of 658 with a yield of 0.5% and a specific activity of 13.8 U/mg of protein were obtained.

Table 4. Purification of pepsinogen from the stomach of albacore tuna

Purification step	Total protein (mg)	Total activity (U)	Specific activity (U/mg)	Purification (fold)	Yield (%)
Crude extract	9989	1235	0.124	1	100
Step 1: Gel filtration					
Sephacryl S-200	1850	475	0.257	2	39
Step 2: Gel filtration					
Sephadex G-50	87	308	3.53	168	25
Step 3: Anion-exchange					
DEAE-cellulose	19	205	10.8	513	17
Step 4: Gel filtration					
Sephadex G-50	10	129	12.8	608	10
Step 5: Anion-exchange					
DEAE-cellulose	0.42	5.9	13.8	658	0.5

*One unit of activity was defined as the amount causing an increase of 1.0 in absorbance at 280 nm per min. Pepsin activity was assayed at pH 2.0, 50 °C for 20 min using hemoglobin as a substrate.

2.4.2 Molecular weight of pepsinogen

Native-PAGE and zymography of purified PG are shown in Figure 5a and 5b, respectively. PG showed a single protein band on native-PAGE. Coincidentally, only one activity zone on the zymogram was noticeable, indicating the purity of the enzyme with pepsin activity developed under acidic condition used for activity staining. PG and its corresponding active form, pepsin, also exhibited a single band on SDS-PAGE under reducing condition (Figure 5c). Molecular weights (MW) of PG and pepsin estimated from the mobilities in SDS-PAGE were 39.9 and 32.7 kDa, respectively. The results suggested that albacore pepsin is the monomeric protein. The MW of PG from albacore tuna was similar to those from polar cod (Arunchalam and Haard, 1985), North Pacific bluefin tuna (Tanji *et al.*, 1988) and smooth hound (Bougatef *et al.*, 2008). A slightly lower MWs of PGs from other fish species has been reported such as sea bream (Zhou *et al.*, 2007), mandarin fish (Zhou *et al.*, 2008), and European eel (Wu *et al.*, 2009a), which had MW ranging from 32 to 38 kDa. After activation of PGs, the MWs of the corresponding pepsins from those fish species ranged from 29 to 32 kDa.

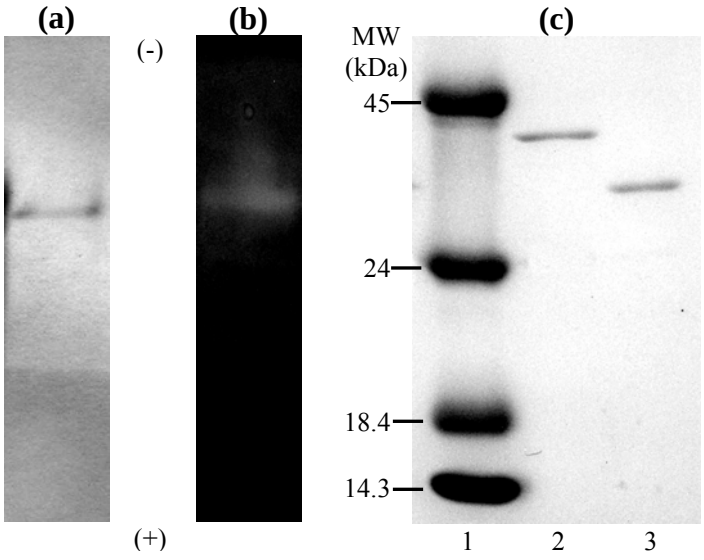

Figure 5. Native-PAGE of purified pepsinogen (PG) from the stomach of albacore tuna (a), zymogram of albacore tuna pepsin (b) and SDS-PAGE of PG and pepsin (c). Lane 1: standard protein markers; lane 2: purified pepsinogen; lane 3: pepsin.

2.4.3 Activation of pepsinogen

Activation of PG was carried out at pH 2 and 4 °C and was monitored by the release of prosegment with the concomitant formation of pepsin (Figure 6). Conversion of pepsinogens to pepsins proceeds autocatalytically at acidic pH by two different pathways, a one-step pathway to release the intact activation segment (prosegment) directly, and a stepwise pathway through a pseudopepsin(s) (Kageyama, 2002). PG from albacore tuna stomach was converted to be active pepsin by a two-step process through an intermediate form (pseudopepsin), which had MW of 36.8 kDa. Tanji *et al.* (2007) found that the MWs of intermediate form of PG1 and PG2 from African coelacanth were 34.1 kDa. The generation of the intermediate form rapidly occurred in a few second during activation. The complete activation was observed after 30-60 min. Activation of PG by two-step process has been reported for PG2 from North Pacific bluefin tuna (Tanji *et al.*, 1988), PG-I from sea bream (Zhou *et al.*, 2007), PG1 and PG2 from African coelacanth (Tanji *et al.*, 2007), PG-I and

PG-II from Mandarin fish (Zhou *et al.*, 2008), as well as PG-I and PG-III from European eel (Wu *et al.*, 2009a). However, the direct conversion of pepsinogen to pepsin in one-step process was also reported for PG3 from North Pacific bluefin tuna (Tanji *et al.*, 1988) and PG from smooth hound (Bougatef *et al.*, 2008). In pepsinogen, the prosegment covers the active-site cleft, and at neutral or weakly alkaline conditions, the inactive zymogen is stabilized through electrostatic interactions between basic amino acid residues in the prosegment and acidic residues in the enzyme moiety of the molecule (Nielsen and Foltmann, 1993). In acidic condition, most carboxyls of pepsinogen are protonated, resulting in weakening of electrostatic interactions. The conformational changes also occur in the activation segment plus the first 13 residues of the pepsin moiety (Kageyama, 2002; Sielecki *et al.*, 1991). The active site is then exposed and the pepsinogen molecule cleaves off its own activation segment automatically (Kageyama, 2002).

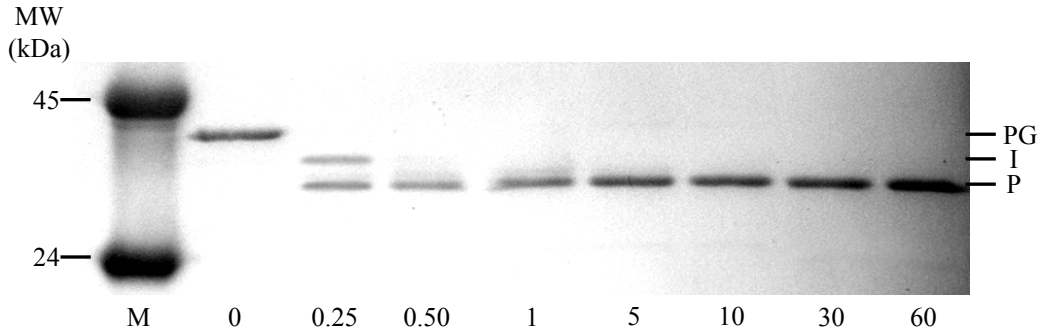

Figure 6. Time courses of the activation of purified pepsinogen (PG) from the stomach of albacore tuna. The activation was carried out at pH 2.0 and 4°C. The conversion reactions were terminated at the time designated and subjected to SDS-PAGE. M: Standard protein markers. PG: pepsinogen; I: intermediate; P: pepsin. The numbers denote the time of activation (min).

2.4.4. N-terminal amino acid sequence of pepsinogen

The N-terminal amino acid sequence of PG from the stomach of albacore tuna was aligned to compare with those of others pepsinogens as depicted in

Figure 7. The N-terminal amino acid sequence of the first 15 amino acids of activation segment of PG was determined to be FHKLPLIKGKTAREE (Figure 7). PG from albacore tuna had the similar N-terminal amino acid sequence to PG2 from North Pacific bluefin tuna (Tanji *et al.*, 1988). The highly conserved amino acid sequence between PGs from albacore tuna and North Pacific bluefin tuna suggested that they were genetically evolved from a common ancestor. PG from albacore tuna showed the sequence homology of 60 and 40%, compared with PG2 from Mandarin fish (Zhou *et al.*, 2008) and African coelacanth (Tanji *et al.*, 2007), respectively. On the other hand, relatively low identities to PGs from smooth hound (Bougatef *et al.*, 2008) and porcine (Nielsen and Foltmann, 1995; Ong and Perlmann, 1968) were observed. The first 15 amino acid of activation segment of PG from albacore tuna contained 5 basic amino acid residues including His2, Lys3, Lys8, Lys10 and Arg13. Basic amino acid residues at positions 3, 8, 10, 13 and 36 in the activation segment have been shown to be important in the stabilization of pepsin moiety at neutral pH (Bougatef *et al.*, 2008; Richter *et al.*, 1998). Therefore, the differences in those basic amino acids between various pepsinogens may govern a conformational change in the activation segment, resulting in the differences in stability and the activation mechanism of the enzyme.

	1				5						10				15
Albacore tuna	F	H	K	L	P	L	I	K	G	K	T	A	R	E	E
Bluefin tuna 2	F	H	K	L	P	L	I	K	G	K	T	A	R	E	E
Coelacanth 1	I	A	K	V	P	L	I	K	E	X	P	L	R	A	I
Coelacanth 2	I	A	K	V	P	L	I	K	E	X	P	L	R	A	I
Coelacanth 3	L	I	S	V	P	L	K	R	F	X	S	I	R	E	S
Bluefin tuna 3	-	I	N	V	P	L	T	R	H	K	S	M	R	E	S
Smooth hound	L	L	R	V	P	L	R	K	G	K	S	T	L	D	V
Bluefin tuna 1	L	L	Q	V	P	L	E	K	G	Q	S	A	R	E	Y
Madarin PG-I	L	K	Q	V	P	L	E	K	G	K	T	A	-	-	-
Madarin PG-II	L	I	Q	V	P	L	E	K	G	K	T	A	R	E	L
Porcine A	L	V	K	V	P	L	V	R	K	K	S	L	R	Q	N
Porcine B	M	E	R	I	I	L	R	K	G	K	S	I	R	E	A

Figure 7. Comparison of N-terminal amino acid sequence of purified pepsinogen (PG) from albacore tuna with other pepsinogens: North Pacific bluefin tuna 1, 2 and 3 (Tanji *et al.*, 1988); coelacanth 1, 2 and 3 (Tanji *et al.*, 2007); smooth hound (Bougatef *et al.*, 2008); mandarin PG-I and PG-II (Zhou *et al.*, 2008); porcine A (Ong and Perlmann, 1968); porcine B (Nielsen and Foltmann, 1995). Amino acid residues different from albacore tuna pepsinogen are boxed.

2.4.5 pH and temperature profile

The effect of pH on the activity of pepsin from the stomach of albacore tuna was determined over a pH range of 1.0-7.0 as shown in Figure 8a. The pepsin was highly active between pH 1.5 to 3.0 with a relative activity more than 80%. The optimal pH for hydrolysis of hemoglobin by pepsin was found at pH 2.0, which was in accordance with those of pepsins obtained from the stomach of bonito (Kubota and Ohnuma, 1970), polar cod (Arunchalam and Haard, 1985), African coelacanth (Tanji *et al.*, 2007), smooth hound (Bougatef *et al.*, 2008), skipjack tuna and tongol tuna (Nalinanon *et al.*, 2008b). Nevertheless, the higher optimal pHs (2.5-3.5) of pepsins from other fish species including North Pacific bluefin tuna (Tanji *et al.*, 1988), sea bream (Zhou *et al.*, 2007), pectoral rattail (Klomklao *et al.*, 2007), Madarin fish (Zhou *et al.*, 2008) and European eel (Wu *et al.*, 2009a), have been reported. Activity of

pepsin generally decreased at pHs higher and lower than the optimal pH. This was possibly due to the conformational changes of enzyme under harsh condition, resulting in the lowered activity (Benjakul *et al.*, 2003; Nalinanon *et al.*, 2008b).

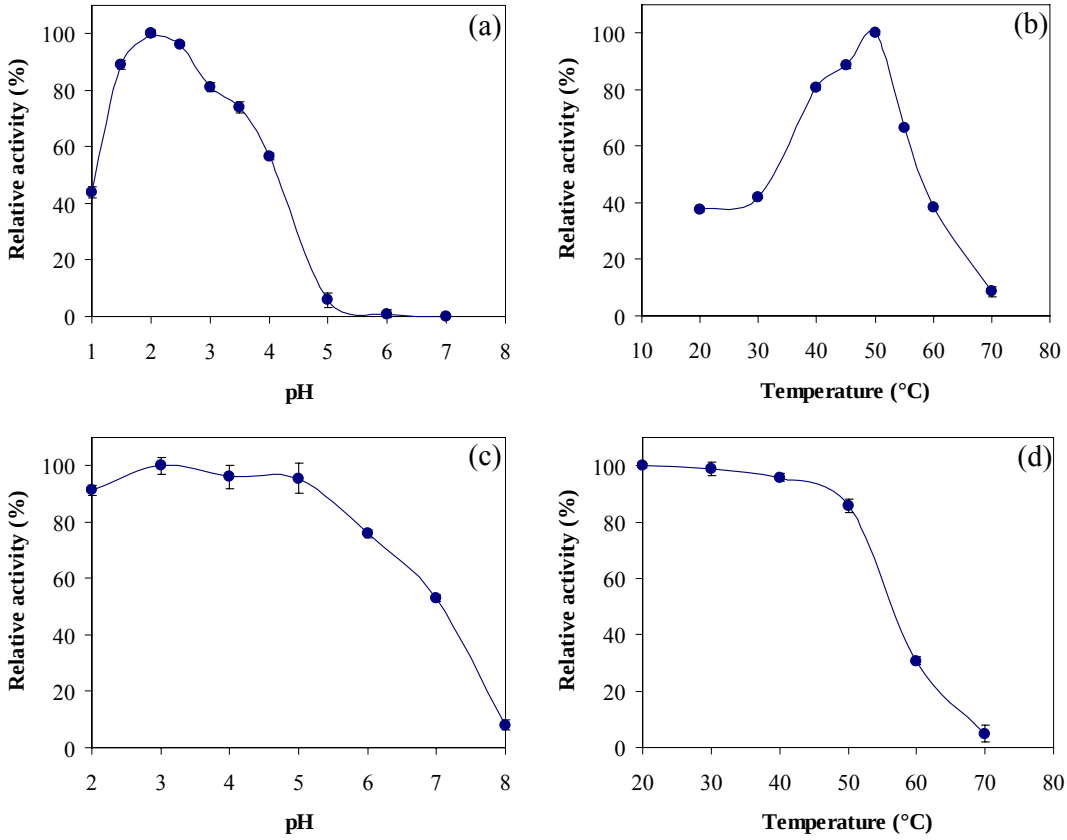

Figure 8. pH (a) and temperature (b) profiles and pH (c) and thermal (d) stability of pepsin from the stomach of albacore tuna. Bars represent the standard deviation (n=3).

Temperature profile of pepsin from the stomach of albacore tuna is depicted in Figure 8b. The optimal temperature of pepsin was 50 °C when hemoglobin was use as a substrate. This optimal temperature was higher than those of pepsins obtained from marine subtropical fish, warm water fish and cold water fish, such as smooth hound (40 °C) (Bougatef *et al.*, 2008), sea bream (45 °C for P-I) (Zhou *et al.*, 2007) and polar cod (37 °C) (Arunchalam and Haard, 1985) and pectoral

rattail (45 °C) (Klomklao *et al.*, 2007), respectively. The varying optimal temperatures of pepsin from fresh water fish including Madarin fish (40-45 °C) (Zhou *et al.*, 2008) and European eel (35-40 °C) (Wu *et al.*, 2009a) have been reported. The differences in optimal temperature might be associated with the differences in enzyme conformation as governed by habitat, environment and genetics (Gildberg, 1988). A sharp decrease in activity of albacore pepsin at temperature above 50 °C was mostly likely owing to thermal denaturation.

2.4.6 pH and thermal stability

The pH stability of pepsin from the stomach of albacore tuna is shown in Figure 8c. Albacore pepsin was stable in the pH range of 2-5, in which the residual activity more than 85% was found. The marked decrease in activity was noticeable at pH above 5. Similar results were reported for orange roughy pepsins (Xu *et al.*, 1996), Monterey acidic enzymes (Castillo-Yañez *et al.*, 2004) and pectoral rattail pepsins (Klomklao *et al.*, 2007). Those enzymes were stable at pH range of 2-6 and became susceptible to activity loss at neutral and alkaline pH. Tanji *et al.* (2007) found that pepsin from African coelacanth at pH 4.5 had 30% of the maximal activity observed at pH 2. Pepsins from cold and temperate water fish are stable in the pH range of 2-5 (Gildberg, 1988), whereas pepsins from warm water species such as bonito are quite stable even under neutral conditions (Kubota and Ohnuma, 1970). The differences in pH stability indicated the different molecular properties including bondings stabilizing the structure as well as enzyme conformation among various species and anatomical location (Klomklao *et al.*, 2007).

Thermal stability of pepsin from the stomach of albacore tuna is shown in Figure 8d. Albacore pepsin was stable up to 50°C with the residual activity more than 85%. Nevertheless, the sharp decrease in activity was noticeable at temperature above 50°C. Relatively low activity was remained at 70°C, suggesting the drastic loss in activity caused by thermal denaturation of pepsin. The result is in agreement with pepsin from dogfish stomach, which was stable at temperature below 50°C (Guerard and Le Gal, 1987). At high temperatures, enzyme possibly underwent denaturation and lost its activity. Klomklao *et al.* (2007) reported that the activity of two pepsins

purified from pectoral rattail was stable up to 40°C and unstable at higher temperatures. Potential number of disulfide linkages, the average hydrophobicity and the amount of intramolecular hydrogen bonds are considered to affect thermostability of enzymes (Gildberg *et al.*, 1990). Tello-Solís and Romero-García (2001) observed the aggregation of porcine pepsin at temperature higher than 50°C. Therefore, the irreversible loss in pepsin activity might be associated with thermal aggregation.

2.4.7 Effect of protease inhibitors and chemicals

Effects of various inhibitors and some chemicals on proteolytic activity of pepsin from the stomach of albacore tuna were determined as shown in Table 5. The proteolytic activity was strongly inhibited by pepstatin A with the concentration range of 0.1-10 μM. E-64, which is specific inhibitor to cysteine proteinase, EDTA, a metalloproteinase inhibitor and soybean trypsin inhibitor, the inhibitor specific to serine proteinase, had no effect on the activity. Pepstatin A, a pentapeptide, is very specific inhibitor, which is able to interact noncovalently with the active site of aspartic proteinases and blocks the accessibility of a substrate to the active site cleft (Fusek and Větvička, 1995; Haard, 1994). Guerard and Le Gal (1987) reported that the activity of dogfish pepsin II was progressively reduced to about 50% of its initial value when pepstatin A at a level of 6×10^{-7} M was added, while a total inhibitory effect was obtained at 10^{-6} M. Pepsins from the stomach of skipjack tuna and tongol tuna were completely inhibited by 1 μM pepstatin A (Nalinanon *et al.*, 2008b). Klomklao *et al.* (2007) reported that the activity of pepsins from the stomach of pectoral rattail was almost inhibited by 0.01 mM pepstatin A . Therefore, the inhibitory results confirmed that purified enzyme was aspartic proteinase, most likely pepsin.

ATP and molybdate, which are activator and inhibitor of cathepsin D, respectively (Pillai and Zull, 1985), had no profound effect on the activity (Table 5). The result was in accordance with Klomklao *et al.* (2007) who reported that ATP and molybdate had no impact on the activity of pepsin from pectoral rattail (Klomklao *et al.*, 2007), skipjack tuna and tongol tuna (Nalinanon *et al.*, 2008b). Pillai and Zull (1985) found that cathepsin D was generally activated by ATP when bovine serum

albumin, hemoglobin, parathyroid hormone and a synthetic octapeptide were used as substrates, whereas pepsin, a homologous aspartic proteinase, was not activated. NaCl and $CaCl_2$ also had no impact on the activity of pepsin. Sánchez-Chiang *et al.* (1987) found that the activity of pepsin I from adult salmon (*Oncorhynchus keta*) towards hemoglobin was increased by approximately 20% in the presence of 5 mM NaCl, whereas the activity of pepsin II was not affected. However, the activities of cod pepsin and porcine pepsin were strongly depressed in the presence of NaCl at the concentration of 5 and 10% (Gildberg *et al.*, 1990). Klomklao *et al.* (2007) found that the activity of both pepsins A and B from pectoral rattail was increased in the presence of 5 mM $CaCl_2$, 5 mM $MgCl_2$ or 5 mM $CoCl_2$. Salts possibly altered the conformation of either pepsin or the substrate used in the way, which impeded or enhanced the binding ability between enzyme and substrate. Albacore pepsin was strongly inhibited by SDS and cysteine. Nalinanon *et al.* (2008b) found that cysteine, a reducing agent, inhibited the pepsin activity of tuna stomach extracts in a dose dependent manner via breaking disulfide bonds of pepsin. Furthermore, SDS, an anionic surfactant, could act as a denaturant by disrupting non-covalent bonds stabilizing the pepsin structure.

Table 5. Effect of various inhibitors and some chemicals on the activity of pepsin from the stomach of albacore tuna*

Inhibitors/Chemicals	Concentration	Relative activity (%)[a]
Control		100
Pepstatin A	0.1 μM	5.27 ± 0.2
	1 μM	ND
	10 μM	ND
E-64	0.1 mM	99.83 ± 0.8
EDTA	2 mM	96.26 ± 1.1
Soybean trypsin inhibitor	0.1 mM	100.0 ± 0.3
ATP	0.5 mM	100.7 ± 4.1
Molybdate	0.5 mM	100.0 ± 5.9
NaCl	5 mM	101.4 ± 4.3
CaCl	5 mM	102.8 ± 4.1
SDS	0.05% (w/v)	ND
Cysteine	50 mM	23.98 ± 5.2

* Enzyme solution was incubated with the same volume of inhibitors or chemicals at 25 °C for 30 min and the residual activity was determined using haemoglobin as a substrate for 20 min at pH 2.0 and 50 °C.

[a] Mean ± SD from triplicate determinations

ND = not detectable

2.5 Conclusions

Pepsinogen from the stomach of albacore tuna was purified to homogeneity. On the basis of biochemical properties, pepsinogen was activated to pepsin at the acidic pH. Resultant pepsin exhibited the proteolytic activity similar to other fish pepsins, particularly from tropical fish. Albacore pepsin could be used as a biotechnological alternative for food processing and other applications.

CHAPTER 3

PURIFICATION AND BIOCHEMICAL PROPERTIES OF PEPSINS FROM THE STOMACH OF SKIPJACK TUNA (*KATSUWONUS PELAMIS*)

3.1 Abstract

Pepsins 1 and 2 from the stomach of skipjack tuna (*Katsuwonus pelamis*) were purified to homogeneity by using a series of chromatographic purification involving DEAE-cellulose, Sephadex G-50 and Sephadex G-75 with increase in purity of 246-fold and 213-fold, respectively. Molecular weights of pepsins 1 and 2 were estimated by SDS-PAGE to be 33.9 and 33.7 kDa, respectively. The N-terminal amino acid sequences of the first 20 amino acids of both isoenzymes were YQDGTEPMTNDADLSYYGVI. The optimal pH and temperature for pepsin 1 were 2.5 and 50 °C, respectively, while pepsin 2 showed optimal activity at pH 2.0 and 45 °C. The activity of two pepsins was stable in the pH range of 2-5 and at temperatures up to 50 °C. The activity of purified pepsins was strongly inhibited by pepstatin A in a dose-dependent manner. SDS and cysteine showed inhibitory effects towards both pepsins. Activity of pepsin 2 was slightly activated by NaCl, but NaCl had no effect on pepsin 1. Pepsins 1 and 2 had high affinity and hydrolytic activity toward hemoglobin with K_m of 54 and 71 μM, respectively. k_{cat} of pepsins 1 and 2 were 38.1 and 44.3 s^{-1}, respectively. Both pepsins effectively hydrolyzed bovine serum albumin, egg white, natural actomyosin from brownstripe red snapper muscle and acid-solubilized collagen from arabesque greenling skin. Nevertheless, the hydrolytic activity was slightly less than that of pepsin from porcine stomach.

3.2 Introduction

Pepsin is the major digestive enzyme in stomach of animals, and is secreted as pepsinogen from chief cells of oxyntic glands located in the stomach wall epithelium (Gildberg, 1988; Kageyama, 2002). In acidic environment, pepsinogen autocatalytically converts to an active form, pepsin (Kageyama, 2002; Nalinanon *et al.*, 2010a; Tanji *et al.*, 2007). Acidification of crude extract is required to activate pepsinogen to pepsin (Chapter 2). Pepsin is composed of a single polypeptide chain of 321 amino acids and has a molecular weight of 35.5 kDa (Whitaker, 1994). Its tertiary structure is stabilized in part by three disulfide bridges and a phosphate linkage. A phosphate group, attached to the hydroxyl group of a seryl residue, can be removed without loss of enzymatic activity (Whitaker, 1994). The enzyme is quite stable in the pH range of 2-5 and rapidly loses activity at pH above 5, due to denaturation (Gildberg, 1988; Nalinanon *et al.*, 2010a; Whitaker, 1994). Pepsin has its primary specificity toward the amino acid residue that furnishes the NH group to the susceptible peptide bond, preferably phenylalanyl, tyrosyl, or tryptophanyl (Whitaker, 1994). Pepsin and pepsinogen from stomach of various fish species such as polar cod (Arunchalam and Haard, 1985), North Pacific bluefin tuna (Tanji *et al.*, 1988), Atlantic cod (Gildberg *et al.*, 1990), sea bream (Zhou *et al.*, 2007), African coelacanth (Tanji *et al.*, 2007), pectoral rattail (Klomklao *et al.*, 2007), smooth hound (Bougatef *et al.*, 2008), Mandarin fish (Zhou *et al.*, 2008), European eel (Wu *et al.*, 2009) and albacore tuna (Nalinanon *et al.*, 2010a) have been purified and characterized.

Thailand is the world's largest producer and exporter of canned tuna. In 2008, Thai canned tuna was exported to several countries with a total volume of 484,894 metric tons and a value of 61,036 million baths (Department of Foreign Trade, 2009). The most commercially important tuna species for the Thai tuna industry is skipjack tuna, followed by yellow-fin tuna and tongol tuna, accounting for over 80% of Thai frozen tuna imports (Department of Foreign Trade, 2009). During canned tuna manufacturing, high amounts of processing wastes such as viscera, head, and bone are discarded. Tuna processing byproducts are normally utilized as fishmeal or pet food, which have a lower market value. There is growing interest in obtaining higher value biochemicals and pharmaceuticals from fishery wastes, notably enzymes.

Of these byproducts, fish viscera can be used as a potential source of various enzymes, particularly proteases (Klomklao *et al.*, 2004; Nalinanon *et al.*, 2008b). Pepsin from the stomach of albacore tuna was isolated and partially purified by aqueous two-phase system (ATPS) (Nalinanon *et al.*, 2009). Fish pepsins have been used for preparation of protein isolate (Pavlisko *et al.*, 1999), extraction of collagen (Nalinanon *et al.*, 2007; Nalinanon *et al.*, 2008b) and gelatin (Nalinanon *et al.*, 2008a). Due to a large amount of skipjack tuna stomach produced during manufacturing, it can serve as a promising source of pepsin for further applications, especially for the digestion of proteins to obtain the high-value products with bioactivity, e.g. nutraceutical properties or flavorant, etc. No information regarding the biochemical characteristics of pepsins from the stomach of skipjack tuna has been reported. Therefore, the objectives of this study were to purify pepsins from skipjack tuna stomach and to characterize some biochemical properties as well as hydrolytic ability towards different protein substrates. For pepsin from tongol tuna, it was not purified and characterized due to the less abundance for tuna processing in Thailand.

3.3 Materials and Methods

3.3.1 Chemicals

Bovine hemoglobin, *β*-mercaptoethanol (βME), *L*-cysteine, adenosine 5′-triphosphate (ATP), molybdate, pepstatin A, ethylenediaminetetraacetic acid (EDTA), soybean trypsin inhibitor, 1-(*L*-trans-epoxysuccinyl-leucylamino)-4-guanidinobutane (E-64) and bovine serum albumin (BSA) were purchased from Sigma Chemical Co. (St. Louis, MO, USA). Folin-Ciocalteu's phenol reagent was obtained from Merck (Darmstadt, Germany). Sephadex G-50 was procured from Pharmacia Biotech (Uppsala, Sweden). Diethylaminoethyl (DEAE)-cellulose was obtained from Whatman (Maidstone, UK). Sephadex G-75 was purchased from GE Healthcare UK Limited (Buckinghamshire, UK). Sodium dodecyl sulfate (SDS), Coomassie Blue R-250, and *N,N,N′,N′*-tetramethyl ethylene diamine (TEMED) were procured from Bio-Rad Laboratories (Hercules, CA, USA).

3.3.2 Preparation of skipjack tuna stomach

Frozen skipjack tuna (*K. pelamis*), off-loaded approximately a month after capture and freezing, were obtained from Tropical Canning (Thailand) Public Co., Ltd., Songkhla, Thailand. After thawing, the internal organs from 50 fish were collected and packed in polyethylene bag, kept in ice with a solid/ice ratio of 1:2 (w/v) and transported to the Department of Food Technology, Prince of Songkla University, Hat Yai within 30 min. Upon arrival, internal organs were excised and the stomach was collected and placed in a polyethylene bag. The collected stomach was stored at -20 °C until use.

3.3.3 Preparation of crude extract

Frozen stomach was thawed using running water (26-28 °C) until the core temperature reached -2 to 0 °C. The sample was cut into pieces with a dimension of 0.5 × 0.5 cm^2. Sample was finely ground in liquid nitrogen using a National Model MX-T2GN blender (Taipei, Taiwan). Sample was then lyophilized using a SCANVAC CoolSafe™ freeze-dryer (CoolSafe 55, ScanLaf A/S, Lynge, Denmark) and the dry powder was referred to as 'stomach powder'.

To prepare the crude extract, stomach powder was suspended in 20 mM sodium phosphate buffer (pH 7.0) containing 1 mM PMSF at a ratio of 1:9 (w/v) and stirred continuously at 4 °C for 3 h. The suspension was centrifuged for 20 min at 4 °C at 20,000 ×*g* using a refrigerated centrifuge (H-2000B, Kokusan, Tokyo, Japan) to remove the tissue debris. The supernatant was collected and lyophilized using an EYELA FDU-1200 freeze-dryer (Tokyo Rikakikai Co., Ltd., Tokyo, Japan). Before use, the dry extract (10 g) was dissolved with 90 mL of 20 mM sodium phosphate buffer (pH 7.0), followed by centrifugation for 15 min at 4 °C at 20,000 ×*g*. The supernatant was collected and referred to as "crude extract".

3.3.4 Purification of skipjack tuna pepsins

All steps of purification were carried out at 4 °C. Crude extract was adjusted to pH 4 with 1 M HCl. The mixture was allowed to stand for 30 min. The acidified crude extract was then centrifuged at 20,000 ×*g* for 15 min. To fully activate pepsin, the obtained supernatant was adjusted to pH 2 with 1 M HCl and gently stirred for 30 min. The solution was subjected to centrifugation as described previously prior to dialysis against 60 volumes of 20 mM sodium phosphate buffer (pH 5.0), referred to as 'starting buffer; SB'. Prepared solution was applied onto DEAE-cellulose column (2.2 × 35 cm), previously equilibrated with SB. After the column was washed with SB until A_{280} was below 0.05, the elution was performed with a linear gradient of 0-0.5 M NaCl in SB at a flow rate of 0.5 mL/min. Fractions of 4 mL were collected and those with proteolytic activity were pooled. Two activity peaks (pepsins 1 and 2) were obtained and pooled fractions from each peak were dialyzed with 60 volumes of SB for 12 h and then concentrated by lyophilization. Both lyophilized fractions were further chromatographed on Sephadex G-50 column (3.9 × 70 cm) equilibrated with approximately two bed volumes of SB. Elution was carried out with the same buffer at the flow rate of 0.5 mL/min. Fractions (4.5 mL) were collected and those with proteolytic activity were pooled and lyophilized. Lyophilized Sephadex G-50 fraction was dissolved in distilled water and dialyzed against 60 volumes of SB for 12 h. The dialysate was then subjected to DEAE-cellulose column and the enzyme was eluted with a linear gradient of 0-0.3 M NaCl. Fractions of 4.5 mL, with proteolytic activity, were pooled and dialyzed against 60 volumes of SB for 12 h prior to lyophilization. Lyophilized DEAE-cellulose fraction was dissolved in SB prior to loading onto Sephadex G-75 (3.2 × 56 cm) column equilibrated with approximately two bed volumes of SB. The proteases were eluted with SB at the flow rate of 0.5 mL/min and fractions of 4.5 mL were collected. Those fractions with proteolytic activity were pooled and lyophilized. The lyophilized fraction was dissolved in SB and re-chromatographed onto Sephadex G-75 column, followed by the elution with SB at the flow rate of 0.25 mL/min. Fractions (2.5 mL) were collected and those with proteolytic activity were pooled and lyophilized.

Pepsin from porcine stomach mucosa (EC 3.4.23.1) (750 units/mg dry matter, Sigma Chemical Co., St. Louis, MO, USA) was partially purified by 2 steps of purification using DEAE-cellulose column and Sephadex G-75 column as described previously.

3.3.5 Protein determination

Protein concentration was determined by measuring the absorbance at 280 nm or by the method of Lowry *et al.* (1951) using bovine serum albumin as a standard.

3.3.6 Assay of proteolytic activity

Proteolytic activity was determined using hemoglobin as a substrate according to the method of Nalinanon *et al.* (2008b) with some modifications. To initiate the reaction, 200 μL of enzyme solution suitably diluted were added into the assay mixture containing 200 μL of 2% hemoglobin, 200 μL of distilled water and 600 μL of reaction buffer. The reaction was conducted at pH 2.0 and 50 °C for 20 min. To terminate enzymatic reaction, 200 μL of 50% (w/v) trichloroacetic acid (TCA) were added. Unhydrolyzed protein substrate was allowed to precipitate for 1 h at 4 °C, followed by centrifuging at 15,000 × g for 10 min using a KUBOTA 3630 centrifuge (SiGMA Laborzentrifugen, Osterode am Harz, Germany). The oligopeptide content in the supernatant was measured at 280 nm. One unit of activity was defined as the amount causing an increase of 1.0 in absorbance at 280 nm per min. A blank was run in the same manner, except that the enzyme was added into the reaction mixture after the addition of 50% (w/v) TCA.

3.3.7 SDS-polyacrylamide gel electrophoresis (SDS-PAGE) and native-PAGE

SDS-PAGE was performed according to the method of Laemmli (1970). Protein solutions were mixed at 1:1 (v/v) ratio with the SDS-PAGE sample

buffer (0.125 M Tris-HCl, pH 6.8; 4% SDS; 20% glycerol; 10% βME) and boiled for 3 min. The samples (15 μg protein) were loaded onto the gel made of 4% stacking and 12.5% or 12% or 7.5% separating gels and subjected to electrophoresis at a constant current of 15 mA per gel using a AE-6440 electrophoresis apparatus (Atto Co., Tokyo, Japan). After electrophoresis, the gels were stained with 0.1% Coomassie brilliant blue R-250 in 50% methanol and 7% acetic acid and destained with 7% acetic acid. Protein standards, including egg albumin (45.0 kDa), bovine pancreatic trypsinogen (24.0 kDa), bovine milk β-lactoglobulin (18.4 kDa) and egg-white lysozyme (14.3 kDa) were used as molecular weight marker. Gels were imaged using a Canon image scanner (CanoScan LiDE 25, Canon Inc., Tokyo, Japan) and band intensities were quantified with the public domain digital analysis software, ImageJ (ImageJ 1.42q, National Institutes of Health, Bethesda, MD, USA). Native-PAGE was performed in the same manner, except SDS and βME were omitted from the system.

3.3.8 Analysis of amino acid sequence

To analyze the N-terminal sequence, purified pepsins were electroblotted onto polyvinylidenedifluoride (PVDF) membrane (ProBlott®, Applied Biosystems, Foster City, CA, USA) after SDS–PAGE. The amino acid sequence of pepsins was analyzed using a protein sequencer, Procise 492 (Perkin Elmer, Foster City, CA, USA).

3.3.9 Biochemical properties

3.3.9.1 pH and temperature profile

To study pH profile, proteolytic activity of purified pepsins was measured at 50 °C using hemoglobin as a substrate at different pHs (pHs 1.0, 1.5 and 2.0 using 20 mM maleate buffer and pHs 2.0, 2.5, 3.0, 3.5, 4.0, 5.0, 6.0, 7.0 and 8.0 using McIlvaine's buffer (0.2 M Na-phosphate and 0.1 M Na-citrate)). For temperature profile, the assay was performed at various temperatures (20, 30, 40, 45, 50, 55, 60 and 70 °C) at pH 2.0.

3.3.9.2 pH and thermal stability

For pH stability study, purified pepsin solutions were incubated at pHs 2.0, 3.0, 4.0, 5.0, 6.0, 7.0 and 8.0 using McIlvaine's buffer at room temperature for 30 min. Residual activity was then determined using hemoglobin as a substrate at pH 2.0 and 50 °C for 20 min. For thermal stability, the purified pepsins were incubated at different temperatures (20, 30, 40, 50, 60 and 70 °C) for 30 min before rapid cooling using iced water. The residual activity was then assayed as previously described.

3.3.9.3 Effect of some protease inhibitors

Purified pepsin solutions (200 μL) were mixed with 200 μL of single protease inhibitor to obtain the final designated concentration (1 μM for pepstatin A, 0.1 mM for E-64, 0.1 mM for soybean trypsin inhibitor and 2 mM for EDTA). Mixtures were incubated at room temperature for 30 min. The residual activity was measured at pH 2.0 and 50 °C. Percent inhibition was then calculated. To study the inhibitory effect of pepstatin A towards pepsins, different molar ratios of pepstatin A to pepsin (0/1, 0.1/1, 0.25/1, 0.5/1, 1/1, 2/1, 4/1 and 10/1; mol/mol) were used. The mixtures of pepsin and pepstatin A were prepared and the residual activity was determined at pH 2.0 and 50 °C.

3.3.9.4 Effect of some chemicals

Different chemicals (200 μL) were mixed with pepsin solution (200 μL) to obtain the concentration designated (0.5 mM for ATP and molybdate; 5 mM for NaCl and $CaCl_2$; 0.05 % (w/v) for SDS and 50 mM for *L*-cysteine). The mixtures were kept at room temperature for 30 min. The remaining activity was determined at pH 2 and 50 °C and the relative activity was calculated.

3.3.9.5 Kinetic studies

The activity was assayed with different final concentrations of hemoglobin ranging from 1 to 300 μM. The final pepsin concentration for the assay was 0.02 mg/mL. The determinations were repeated twice and the respective kinetic parameters including V_{max} and K_m were evaluated by plotting the data on a

Lineweaver-Burk double-reciprocal graph (Lineweaver and Burk, 1934). Values of turnover number (k_{cat}) were calculated from the following equation: $k_{cat} = V_{max}/[E]$, where [E] is the active enzyme concentration and V_{max} is the maximal velocity.

3.3.9.6 Hydrolysis of different protein substrates by pepsins

BSA, egg white, natural actomyosin (NAM) from brownstripe red snapper (*Lutjanus vitta*) muscle and acid-solubilized collagen (ASC) from arabesque greenling (*Pleurogrammus azonus*) skin were used as protein substrates. NAM and ASC were prepared following the methods of Benjakul *et al.* (1997) and Nalinanon *et al.* (2007), respectively. To hydrolyze the proteins, pepsins (1.6 units) were added to 2 mL of reaction mixture, containing 4 mg of different protein substrates in McIlvaine's buffer, pH 2.0, previously incubated at 50 °C for 10 min. The hydrolysis was conducted by incubating the mixture at 50 °C for 30 min. The control was performed by incubating the reaction mixture at 50 °C for 30 min without the addition of pepsin. At the designated time, 200 μL of reaction mixture was taken and mixed with 100 μL of 1 N NaOH containing 1% (w/v) SDS in order to terminate pepsin activity. The mixture was further mixed with 100 μL of SDS-PAGE sample buffer and boiled for 1 min. The reaction mixture was analyzed by SDS-PAGE using 12% or 7.5% separating gel and 4% stacking gel as previously described.

3.3.10 Statistical analysis

Experiments were run in triplicate. All data were subjected to Analysis of Variance (ANOVA) and the differences between means were evaluated by Duncan's multiple range test. For pair comparison, T-test was used. SPSS statistic program (SPSS 11.0 for Windows, SPSS Inc., Chicago, IL, USA) was used for data analysis.

3.4 Results and Discussion

3.4.1 Purification of skipjack tuna pepsins

Purification of pepsins from the stomach of skipjack tuna is summarized in Table 6. The purification of pepsins was started by adjusting pH of the crude extract to pH 4 and 2, respectively. After acidification, an activity yield of approximately 111% was obtained, and 73.4% of proteins, mostly contaminating proteins, were removed. Acidification was used to remove other protein contaminants in stomach extract, leading to the higher purity of pepsin (Gildberg and Raa, 1983; Klomklao *et al.*, 2007). Increase in purity of 4-fold was obtained after acidification process. Acidified fraction was then subjected to ion-exchange chromatography using DEAE-cellulose. Two distinct peaks with proteolytic activity eluted by 0.07 and 0.15 M NaCl were designated as pepsins 1 and 2, respectively (Figure 9). Purity of pepsins 1 and 2 was increased by 19 and 20 folds, respectively. Both DEAE-cellulose fractions were subsequently chromatographed onto Sephadex G-50 column. . It was noted that 99.7% of protein, mainly contaminating proteins, were removed after gel filtration using Sephadex G-50 column. The results showed that DEAE-cellulose and Sephadex G-50 chromatographies effectively removed the contaminants from the crude extract. However, some protein contaminants were still retained as examined by SDS-PAGE (data not shown). In order to purify pepsins to homogeneity, Sephadex G-50 fractions were concentrated and re-chromatographed using DEAE-cellulose column, followed by Sephadex G-75 column twice. After the final purification step, purification fold of 246 and 213 with a yield of 2.1% and 4.2% were obtained for pepsins 1 and 2, respectively. Specific activity of 93.5 and 81.1 units/mg of protein were found for pepsins 1 and 2, respectively. The homogeneity of purified pepsins was examined by SDS–PAGE. Pepsins 1 and 2 showed single band on SDS–PAGE under reducing conditions (Figure 10). Molecular weights (MW) of pepsins 1 and 2 estimated from the mobilities in SDS-PAGE were 33.9 and 33.7 kDa, respectively. Based on native-PAGE, single band of each pepsin was observed (data not shown), confirming the purity of pepsin obtained. MWs of pepsins from the stomach of skipjack tuna were similar to those of pepsins or acid proteases from the

stomach of Mandarin fish (Zhou *et al.*, 2008), sea bream (Zhou *et al.*, 2007), European eel (Wu *et al.*, 2009), smooth hound (Bougatef *et al.*, 2008), African coelacanth (Tanji *et al.*, 2007), albacore tuna (Nalinanon *et al.*, 2010), pectoral rattail (Klomklao *et al.*, 2007) and orange roughy (Xu *et al.*, 1996), which had MW ranging from 30 to 35 kDa. Although the MW of two pepsin isoforms from skipjack tuna are quite similar, some differences in enzymatic characteristics, especially their binding properties to ion-exchange column, DEAE-cellulose, were noticeable.

Table 6. Purification of pepsins from the stomach of skipjack tuna

Purification step	Total protein (mg)	Total activity (units)*	Specific activity (units/mg)	Purification (fold)	Yield (%)
Crude extract	6250	2383	0.381	1	100
Acidification	1657	2655	1.60	4	111
DEAE-cellulose (1^{st})					
Pepsin 1	44.1	316	7.17	19	13
Pepsin 2	57.4	438	7.63	20	18
Sephadex G-50					
Pepsin 1	7.77	155	19.9	52	6.5
Pepsin 2	9.67	170	16.0	42	7.2
DEAE-cellulose (2^{nd})					
Pepsin 1	2.17	121	55.7	146	5.1
Pepsin 2	2.94	153	52.2	137	6.4
Sephadex G-75 (1^{st})					
Pepsin 1	1.25	92.2	73.7	194	3.9
Pepsin 2	2.55	157	61.7	162	6.6
Sephadex G-75 (2^{nd})					
Pepsin 1	0.53	49.5	93.5	246	2.1
Pepsin 2	1.23	100	81.1	213	4.2

*One unit of activity was defined as the amount causing an increase of 1.0 in absorbance at 280 nm per min. Pepsin activity was assayed at pH 2.0, 50 °C for 20 min using hemoglobin as a substrate.

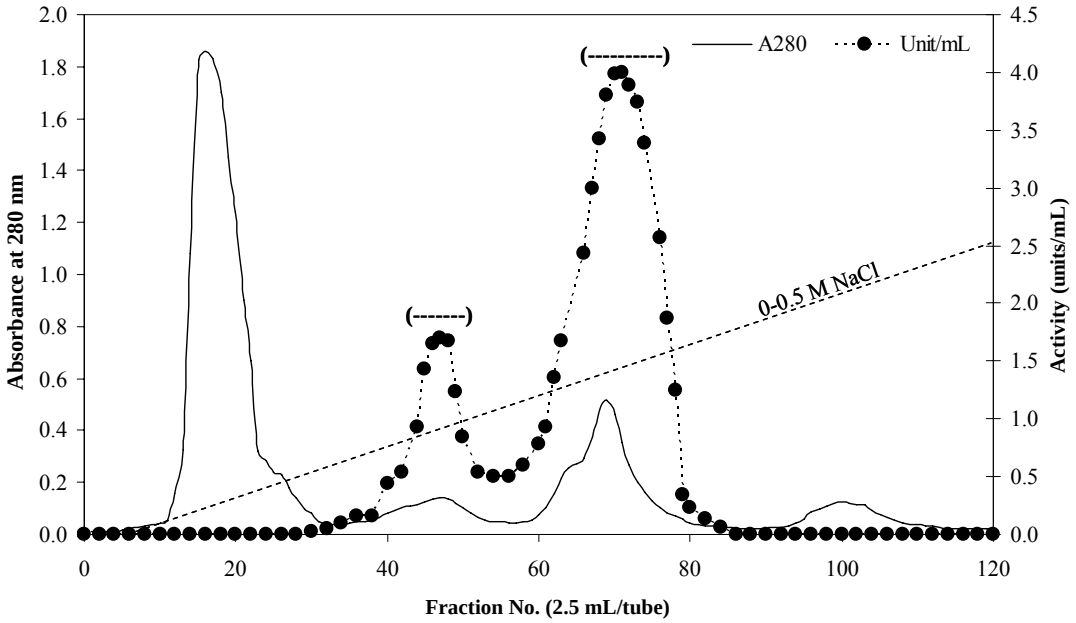

Figure 9. Elution profile of pepsins on DEAE-cellulose column. Acidified extract was dialyzed against SB and applied onto a DEAE-cellulose column. Elution was carried out with a linear gradient of 0–0.5 M NaCl in SB. (---): pooled fractions.

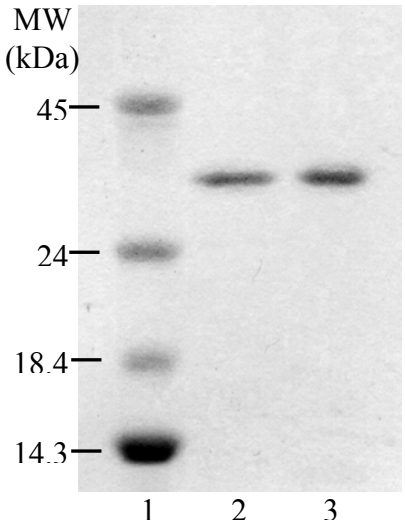

Figure 10. SDS-PAGE of purified pepsins from the stomach of skipjack tuna. Lane 1: standard protein markers; lane 2: pepsin 1; lane 3: pepsin 2.

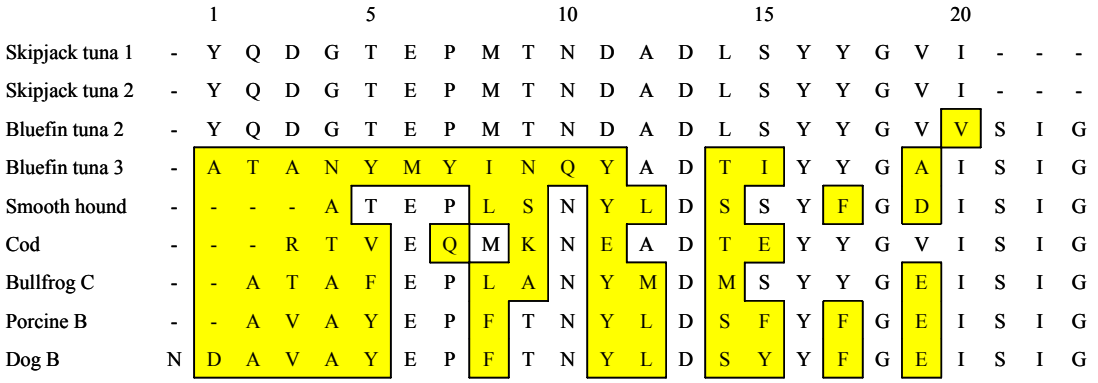

Figure 11. Comparison of N-terminal amino acid sequence of purified pepsins from the stomach of skipjack tuna with other pepsins: North Pacific bluefin tuna pepsin 2 and 3 (Tanji *et al.*, 1988); smooth hound pepsin (Bougatef *et al.*, 2008); cod pepsin (Karlsen *et al.*, 1998); bullfrog pepsin C (Yakabe *et al.*, 1991); porcine pepsin B (Hartsuck *et al.*, 1992) and dog pepsin B (Narita *et al.*, 2002). Amino acid residues different from skipjack tuna pepsin are boxed.

3.4.2 N-terminal amino acid sequence of pepsins

N-terminal amino acid sequence of pepsins from the stomach of skipjack tuna was aligned to compare with those of other pepsins as depicted in Figure 11. The same N-terminal amino acid sequence of the first 20 amino acids, YQDGTEPMTNDADLSYYGVI, was found for pepsins 1 and 2. The result revealed that two pepsins from skipjack tuna stomach might shared high identity. Based on the first 20 amino acids, pepsins from skipjack tuna showed high degree of sequence homology (95%), compared with pepsin 2 from North Pacific bluefin tuna (Tanji *et al.*, 1988). The highly conserved amino acid sequence between pepsins from skipjack tuna and North Pacific bluefin tuna suggested that they were genetically evolved from a common ancestor. According to the first 20 amino acids, pepsins from skipjack tuna showed the sequence homology of 50% and 45%, compared with pepsin from cod (Karlsen *et al.*, 1998) and smooth hound (Bougatef *et al.*, 2008), respectively. Relative low homology of skipjack tuna pepsins in comparison with pepsin B from

porcine (Hartsuck *et al.*, 1992) and dog (Narita *et al.*, 2002) and pepsin C from bullfrog (Yakabe *et al.*, 1991) was observed.

3.4.3 pH and temperature profile

The effect of pH on the activity of pepsins from the stomach of skipjack tuna was determined over a pH range of 1.0-8.0 as shown in Figure 12a. Pepsins 1 and 2 were highly active between pH 1.5 to 3.0. The optimal pH for hydrolysis of hemoglobin by pepsins 1 and 2 was 2.5 and 2.0, respectively. The optimum pH values of both pepsins were in accordance with those of pepsins obtained from the stomach of other fish species including bonito (Kubota and Ohnuma, 1970), polar cod (Arunchalam and Haard, 1985), North Pacific bluefin tuna (Tanji *et al.*, 1988), African coelacanth (Tanji *et al.*, 2007), sea bream (Zhou *et al.*, 2007), pectoral rattail (Klomklao *et al.*, 2007), smooth hound (Bougatef *et al.*, 2008), skipjack tuna and tongol tuna (Nalinanon *et al.*, 2008b), Mandarin fish (Zhou *et al.*, 2008), European eel (Wu *et al.*, 2009) and albacore tuna (Nalinanon *et al.*, 2010), in which optimal pH ranged from 2.0 to 3.5. No pepsin activity was detected at the pH range of 6-8. Denaturation causing the loss in activity was very severe in neutral–alkaline pH ranges. This was possibly due to the conformational changes of enzyme under harsh condition, resulting in the lowered activity or complete loss in activity (Nalinanon *et al.*, 2010).

Temperature profiles of pepsins from the stomach of skipjack tuna is depicted in Figure 12b. The optimal temperature of pepsins 1 and 2 was 50 and 45 °C, respectively, when hemoglobin was used as a substrate. The different temperature optima (40 and 55 °C) between two pepsins from sardine have also been reported (Noda and Murakami, 1981). The optimal temperature of pepsin 1 from skipjack tuna was in accordance with those of pepsins obtained from sea bream (P-II, P-III and P-IV) (Zhou *et al.*, 2007), tongol tuna (Nalinanon *et al.*, 2008b) and albacore tuna (Nalinanon *et al.*, 2010). The varying optimal temperatures of pepsin from other marine fish species which inhabit in different temperature zones such as tropical fish: sea bream (45-50 °C) (Zhou *et al.*, 2007), subtropical fish: smooth hound (40 °C) (Bougatef *et al.*, 2008) and cold water fish: polar cod (37 °C) (Arunchalam and

Haard, 1985) and pectoral rattail (45 °C) (Klomklao *et al.*, 2007) have been reported. The optimal temperatures of pepsin from fresh water fish including Mandarin fish (40-45 °C) (Zhou *et al.*, 2008) and European eel (35-40 °C) (Wu *et al.*, 2009) were also reported. In general, pepsins from colder-water fish had the lower temperature optima and were more heat-labile than those of animals from the warmer environment (Arunchalam and Haard, 1985; Gildberg and Raa, 1983; Squires *et al.*, 1986). The differences in optimal temperature might be associated with the differences in enzyme conformation and bonds stabilizing the enzyme structure as governed by habitat, environment and genetics (Gildberg, 1988; Nalinanon *et al.*, 2010; Nalinanon *et al.*, 2008b). The sharp decrease in activity of both pepsins from skipjack tuna at temperature above 50 °C was observed, mainly due to thermal denaturation of pepsins.

3.4.4 pH and thermal stability

Pepsins 1 and 2 from the stomach of skipjack tuna were stable in the pH range of 2-5, in which the residual activity more than 80% was found (Figure 12c). The decrease in activity of both pepsins was noticeable at pH 6 and pepsins became more susceptible to activity losses at neutral and alkaline pH. Similar result was reported for albacore tuna pepsin (Nalinanon *et al.*, 2010). Orange roughy pepsins (Xu *et al.*, 1996), Monterey acidic enzymes (Castillo-Yañez *et al.*, 2004) and pectoral rattail pepsins (Klomklao *et al.*, 2007) exhibited the stability at pH range of 2-6. Gildberg (1988) reported that pepsins from cold and temperate water fish were stable in the pH range of 2-5, whereas pepsins from warm water species such as bonito were quite stable even in neutral conditions (Kubota and Ohnuma, 1970). The differences in pH stability indicated the different molecular properties including bondings stabilizing the structure as well as enzyme conformation among various species and anatomical location (Klomklao *et al.*, 2007).

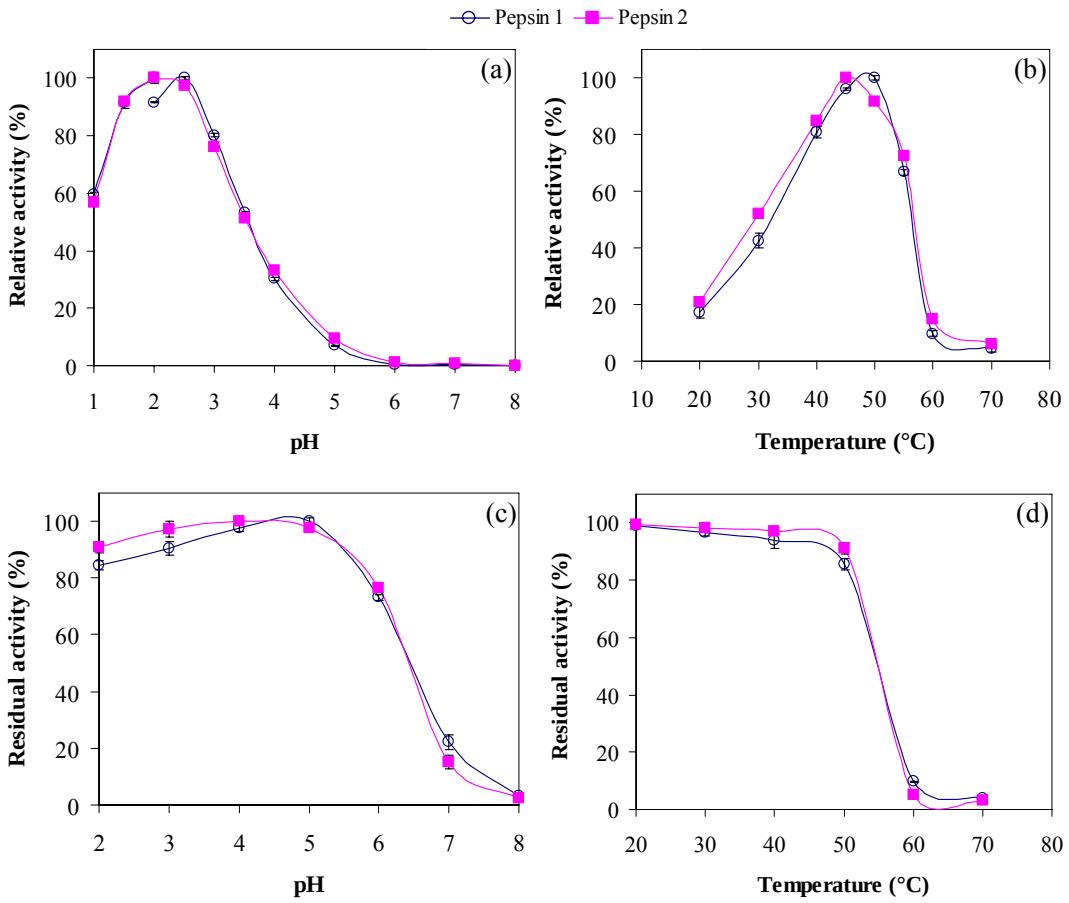

Figure 12. pH (a) and temperature (b) profiles and pH (c) and thermal (d) stabilities of purified pepsins from the stomach of skipjack tuna.

Thermal stability of pepsins 1 and 2 from the stomach of skipjack tuna is depicted in Figure 12d. The activity of both pepsins was stable up to 50 °C with the residual activity more than 85%. After being heated at 60 °C for 30 min, activities of pepsins 1 and 2 were decreased to 9.7% and 4.7%, respectively. The result was in agreement with pepsin from dogfish stomach (Guerard and Le Gal, 1987) and albacore tuna (Nalinanon *et al.*, 2010), which was stable at temperature below 50°C. Disulfide linkages, the average hydrophobicity and the amount of intramolecular hydrogen bonds are considered to affect thermostability of enzymes (Gildberg *et al.*, 1990).

3.4.5 Effect of protease inhibitors and some chemicals

The effects of various inhibitors and some chemicals on activity of pepsins 1 and 2 from the stomach of skipjack tuna were determined (Table 7). The proteolytic activity of both pepsins was strongly inhibited by pepstatin A, an aspartic proteinase inhibitor. Pepstatin A, a pentapeptide, is very specific inhibitor that forms multiple, noncovalent interactions with the active site of aspartic proteinases and blocks the accessibility of a substrate to the active site cleft (Fusek and Větvička, 1995; Haard, 1994). On the other hand, E-64, specific inhibitor of cysteine proteinase, soybean trypsin inhibitor, a serine proteinase inhibitor and EDTA, a metalloproteinase inhibitor had negligible effect on the activity of purified pepsins (Table 7). Therefore, the inhibitory results confirmed that purified enzymes belonged to the aspartic proteinase.

Table 7. Effect of various inhibitors and some chemicals on the activity of pepsins from the stomach of skipjack tuna*

Inhibitors/Chemicals	Concentration	Relative activity (%)[a]	
		Pepsin 1	Pepsin 2
Control		100	100
Pepstatin A	1 μM	ND	ND
E-64	0.1 mM	97.6 ± 0.7	98.4 ± 0.8
EDTA	2 mM	101.3 ± 1.4	99.8 ± 1.7
Soybean trypsin inhibitor	0.1 mM	99.4 ± 1.9	99.1 ± 0.3
ATP	0.5 mM	97.3 ± 1.1	99.5 ± 0.8
Molybdate	0.5 mM	100.5 ± 0.6	99.9 ± 4.6
NaCl	5 mM	102.0 ± 1.6	107.4 ± 0.8
$CaCl_2$	5 mM	99.8 ± 0.7	103.4 ± 1.0
SDS	0.05% (w/v)	59.9 ± 1.2	60.5 ± 0.4
Cysteine	50 mM	79.9 ± 5.1	88.3 ± 1.8

* Enzyme solution was incubated with the same volume of inhibitors or chemicals at 25 °C for 30 min and the residual activity was determined using hemoglobin as a substrate for 20 min at pH 2.0 and 50 °C.

[a] Mean ± SD from triplicate determinations

ND = not detectable

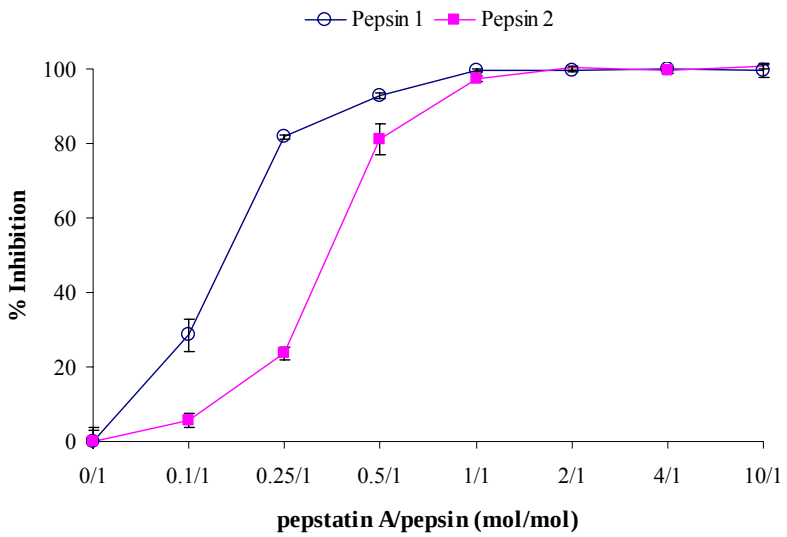

Figure 13. Effect of pepstatin A at different molar ratios on the inhibition of purified pepsins from the stomach of skipjack tuna.

Pepsin 1 from skipjack tuna was specifically bound to pepstatin A with a pepstatin A/pepsin molar ratio of 1:1, whereas pepstatin A completely inhibited pepsin 2 with a 2:1 stoichiometry (Figure 13). The differences in susceptibility of both pepsins toward pepstatin A were also observed in different isoforms of pepsins from the stomach of other fish species such as sea bream (Zhou *et al.*, 2007), European eel (Wu *et al.*, 2009) and Mandarin fish (Zhou *et al.*, 2008). Tanji et al. (Tanji *et al.*, 2007) reported that both pepsins from African coelacanth were nearly completely inhibited by an equimolar amount of pepstatin. Zhou et al. (Zhou *et al.*, 2008) found that pepsins III(a) and III(b) from Mandarin fish were strongly inhibited by pepstatin with a pepstatin/pepsin molar ratio of approximately 1:1. Nevertheless, pepsins I and II were not sensitive to pepstatin A and a 10-fold molar excess of pepstatin A was necessary for the complete inhibition of these two enzymes. Pepsins 1 and 2 from North Pacific bluefin tuna were strongly inhibited by pepstatin, whereas pepsin 3 was less sensitive to pepstatin. A 17-fold molar excess of pepstatin was required for complete inhibition of pepsin 3 (Tanji *et al.*, 1988).

Different chemicals were also used to elucidate their impact on activity of both pepsins (Table 7). ATP and molybdate, activator and inhibitor of cathepsin D (Pillai and Zull, 1985), respectively, did not affect the activity of purified pepsins

from skipjack tuna. Klomklao et al. (2007) reported that ATP and molybdate had no impact on the activity of pepsins from pectoral rattail. Nalinanon et al. (Nalinanon *et al.*, 2010) also found that ATP and molybdate had no profound effect on the activity of albacore tuna pepsin. Cathepsin D is generally activated by ATP when bovine serum albumin, hemoglobin, parathyroid hormone and a synthetic octapeptide were used as substrates, whereas pepsin, a homologous aspartic proteinase, was not activated (Pillai and Zull, 1985). NaCl (5 mM) slightly increased the activity of pepsin 2 from skipjack tuna by 7% ($P < 0.05$) but it had no impact on the activity of pepsin 1. $CaCl_2$ (5 mM) had no profound effect on pepsins activity ($P > 0.05$). Sanchez-Chiang et al. (1987) found that the activity of pepsin I from adult salmon (*Oncorhynchus keta*) towards hemoglobin was increased by approximately 20% in the presence of 5 mM NaCl, whereas the activity of pepsin II was not affected. The activity of both pepsins A and B from the stomach of pectoral rattail was increased in the presence of 5 mM $CaCl_2$, 5 mM $MgCl_2$ or 5 mM $CoCl_2$ (Klomklao *et al.*, 2007). However, the proteolytic activities of pepsins from pectoral rattail was continuously decreased as NaCl concentration increased (0-30%) (Klomklao *et al.*, 2007). Gildberg *et al.* (1990) also reported that all pepsins from Atlantic cod were strongly depressed by the presence of NaCl at the concentrations of 5 and 10%. Salts possibly altered the structural conformation of either pepsin or the substrate used, in the way, which impeded or enhanced the binding ability between both enzyme and substrate (Nalinanon *et al.*, 2010). The presence of 0.05% (w/v) SDS and 50 mM cysteine resulted in the loss in activity of both pepsins by approximately 40% and 12-20%, respectively. The same result was also reported for albacore tuna pepsin (Nalinanon *et al.*, 2010). Nalinanon et al. (2008b) found that cysteine, a reducing agent, inhibited the pepsin activity of tuna stomach extracts in dose dependent manner, mainly via breaking disulfide bonds of pepsin. Furthermore, SDS, an anionic surfactant, could be act as a denaturant by disrupting non-covalent bonds that stabilized the pepsin structure (Nalinanon *et al.*, 2010).

3.4.6 Kinetic study

Kinetic constants, K_m and k_{cat}, for hydrolysis of hemoglobin of purified pepsins from the stomach of skipjack tuna were determined from Lineweaver-Burk plot (Table 8). The apparent K_m of pepsins 1 and 2 were estimated to be 54 and 71 μM, respectively. K_m of pepsin 1 was lower than that of pepsin 2 ($P < 0.05$), suggesting that pepsin 1 had the higher affinity to hemoglobin, compared with pepsin 2. The turnover numbers (k_{cat}) of pepsins 1 and 2 were 38.1 and 44.3 s^{-1}, respectively. The relatively higher k_{cat} value obtained for pepsin 2 indicated that this isoenzyme could contribute to a higher rate of hemoglobin hydrolysis than pepsin 1. The specificity constants (k_{cat}/K_m) of pepsins 1 and 2 were 0.71 and 0.63 $\mu M^{-1} s^{-1}$, respectively, indicating that pepsin 1 had the greater catalytic efficiency for hemoglobin hydrolysis than pepsin 2.

Table 8. Kinetic properties of purified pepsins from the stomach of skipjack tuna stomach for the hydrolysis of hemoglobin

Enzyme	K_m (μM)*	k_{cat} (s^{-1})*	k_{cat}/K_m ($\mu M^{-1} s^{-1}$)
Pepsin 1	54 ± 0.2a	38.1 ± 0.2a	0.71
Pepsin 2	71 ± 0.3b	44.3 ± 0.2b	0.63

K_m, k_{cat} values were determined using hemoglobin as a substrate at pH 2.0 and 50 °C. The final enzyme concentration for the assay was 0.02 mg/mL.

Mean ± SD from triplicate determinations.

* Different letters in the same column indicate the significant differences ($P < 0.05$).

3.4.7 Hydrolysis of different protein substrates by pepsins

The degradation patterns of different protein substrates including BSA, egg white, NAM and ASC hydrolyzed by pepsin from porcine stomach and pepsins 1 and 2 from the stomach of skipjack tuna stomach at 50 °C for 30 min are shown in Figure 14. All protein substrates used were susceptible to pepsin hydrolysis. The degree of hydrolysis of those protein substrates depended upon types of pepsin used. BSA was drastically hydrolyzed by pepsins (Figure 14a). After hydrolysis of BSA by

porcine pepsin, only a protein band (66 kDa) was retained and approximately 89% of BSA was cleaved into small peptides with MW lower than 14.2 kDa. The degradation patterns of BSA hydrolyzed by pepsins 1 and 2 were compared. The peptide fragments with MW of 40-66, 36, 21-29 and 15 kDa were produced when both pepsins were used. However, BSA was slightly more susceptible to hydrolysis by pepsin 2 than pepsin 1 as evidenced by the lower band intensity of proteins with MW of 40-66 kDa. Two major protein components in egg white, conalbumin and ovalbumin were also hydrolyzed by pepsins (Figure 14b). Conalbumin was completely hydrolyzed by both pepsins from skipjack tuna, whereas ovalbumin was more resistant to hydrolysis by those pepsins. Although porcine pepsin showed the greater ability to hydrolyze egg white proteins than pepsins 1 and 2, conalbumin and ovalbumin were still retained to some extents. After pepsin digestion of NAM from brownstripe red snapper muscle, a substantial decrease in myosin heavy chain (MHC) and actin with the concomitant formation of degradation products with MW range of 29-35 kDa as well as those having MW lower than 14.2 kDa was observed (Figure 14c). MHC and actin are the main components of myofibrillar proteins in NAM, followed by troponin and tropomyosin, respectively. The result revealed that MHC and actin were more susceptible to pepsins 1 and 2 digestion, compared with porcine pepsin treatment as indicated by slightly higher remaining MHC and actin after hydrolysis by the latter. Troponin T and tropomyosin were also hydrolyzed to some extent. Degradation patterns of ASC from the skin of arabesque greenling digested by different pepsins are shown in Figure 14d. After pre-heated at 50 °C for 10 min, ASC was readily hydrolyzed by all pepsins tested, particularly by porcine pepsin. No high molecular weight component (HMC), γ and β chains were retained in ASC after skipjack tuna pepsins digestion. The α-chains were also hydrolyzed to a high degree. Coincidentally, peptide fragment with MW of 62.6 kDa and other degradation peptides were produced. The α-components (α1 and α2) of ASC were almost completely hydrolyzed by porcine pepsin. These results revealed that cross-linked components (HMC, β- and γ-components) as well as α-components of ASC could be hydrolyzed by pepsins from skipjack tuna stomach as well as porcine pepsin. However, the hydrolysis of all protein substrates by porcine pepsin was more pronounced than pepsins from skipjack tuna stomach. Additionally, pepsin 2

exhibited slightly higher effectiveness for hydrolysis of those protein substrates than pepsin 1. Therefore, skipjack tuna pepsins can be used for hydrolysis of proteins for further application, especially for the production of protein hydrolysates.

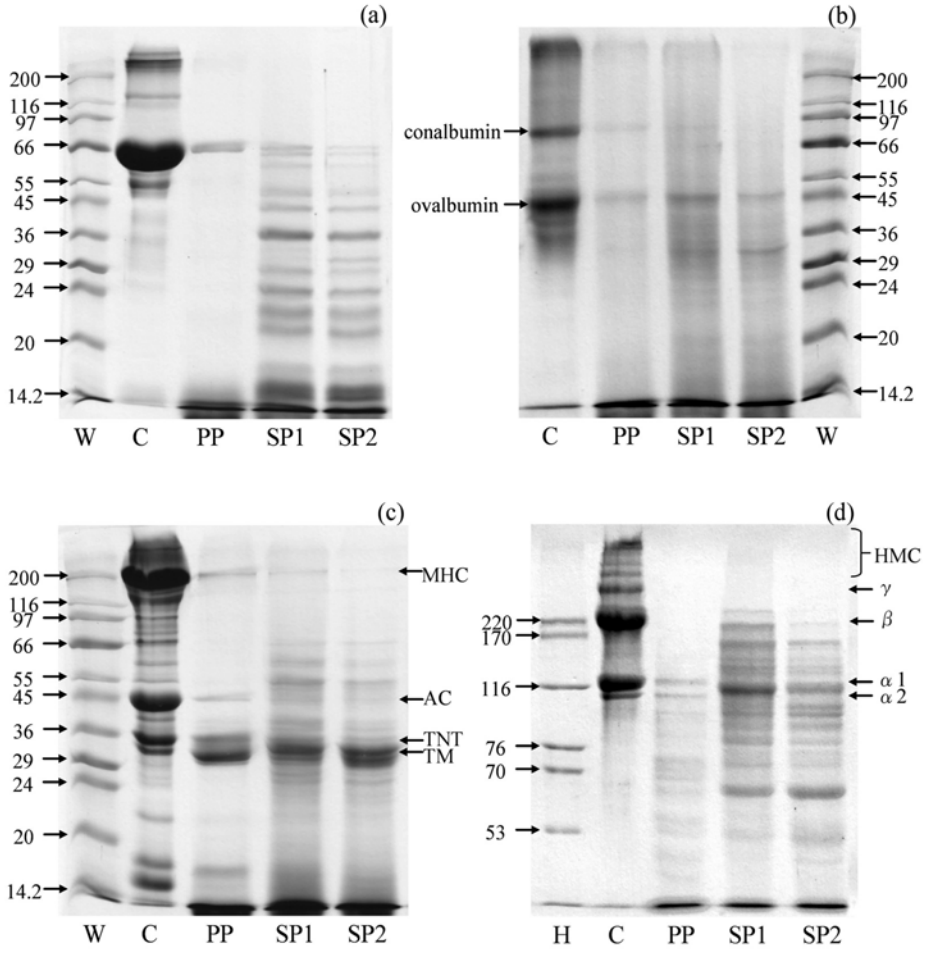

Figure 14. Hydrolysis of BSA (a), egg white (b), natural actomyosin (c) and acid-solubilized collagen (d) by porcine pepsin (PP) and purified pepsin 1 (SP1) and pepsin 2 (SP2) from skipjack tuna stomach at 50 °C for 30 min. W, wide range MW protein marker; H, high MW protein marker; C, control (protein sample incubated without enzyme addition); MHC, myosin heavy chain; AC, actin; TNT, troponin T; TM, tropomyosin; HMC, high molecular weight components. SDS-PAGEs were performed using 12% separating gel for all protein substrates (a, b, c) except 7.5% separating gel for acid-solubilized collagen (d).

3.5 Conclusion

Pepsins 1 and 2 from the stomach of skipjack tuna were purified to homogeneity. Based on biochemical characteristics, they were consistent with those of pepsins from other species. Skipjack tuna pepsins could be effectively used for hydrolysis purposes for food processing and other applications.

CHAPTER 4

PARTITIONING OF PROTEASE FROM STOMACH OF ALBACORE TUNA (*THUNNUS ALALUNGA*) BY AQUEOUS-TWO PHASE SYSTEMS

4.1 Abstract

Partitioning of protease from stomach of albacore tuna using an aqueous two-phase system (ATPS) was investigated. The best ATPS conditions for protease partitioning from stomach extract (SE) and acidified counterpart (ASE) were 25% PEG1000-20% $MgSO_4$ and 15% PEG2000-15% $MgSO_4$, which increased the purity by 7.2-fold and 2.4-fold with the recovered activity of 85.7% and 89.1%, respectively. Electrophoretic study revealed that SE had a major protein with a molecular weight (MW) of 40.6 kDa, while protein with MW of 32.7 kDa was predominant in ASE and ATPS fractions. Pepsinogen in SE might be activated to pepsin by acidification and partitioning process. SE was quite stable at 0 °C and 4 °C up to 14 days. The loss in protease activity in ASE and selected ATPS fractions was more pronounced when storage time and temperature increased. Therefore, ATPS can be effectively used to recover and purify protease from albacore tuna stomach.

4.2 Introduction

Albacore tuna (*Thunnus alalunga*) is an important raw material used for the production of canned tuna in Thailand (Nalinanon *et al.*, 2008b). Large volumes of raw tuna go through the canning process, by which about two-thirds of whole fish is utilized (Klomklao *et al.*, 2005). Thus, high amounts of processing wastes such as viscera, head, and bone are discarded during canned tuna manufacturing. Fish viscera is a potential source for recovering enzymes such as proteinases that may have some unique properties for industrial applications, e.g. in the detergent, food, pharmaceutical, leather and silk industries (Klomklao *et al.*, 2005). Stomach protease, especially pepsin can be recovered and used, mainly for hydrolysis purposes. Fish pepsin has been used for preparation of protein isolate (Pavlisko *et al.*, 1999), extraction of collagen (Nalinanon *et al.*, 2007; Nalinanon *et al.*, 2008b) and gelatin (Nalinanon *et al.*, 2008a).

Nowadays, the efficient and economical downstream processes for the partitioning and purification of biomolecules that give high yield and high purity of the product have been demanded by industries. Partitioning in aqueous two-phase system (ATPS) has been shown to be powerful for separating and purifying the mixtures of proteins (Bensch *et al.*, 2007; Tanuja *et al.*, 1997). ATPS forms readily upon mixing aqueous solutions of two hydrophilic polymers, or of a polymer and a salt, above a certain threshold concentration (Spelzini *et al.*, 2005; Tubío *et al.*, 2007). In general, ATPS yields a specific environment suitable for maintaining enzymes in their native structure and for concentration/purification by means of selective partitioning of the enzyme to one of the phases (Tanuja *et al.*, 1997). Coincidentally, ATPS can remove undesirable enzymes/proteins, unidentified polysaccharides and pigments that are present in the system (Srinivas *et al.*, 2002; Tanuja *et al.*, 1997). ATPS can be scaled up and has the possibility for continuous steady state operation. Additionally, the inexpensive materials that form this system can be recycled (Spelzini *et al.*, 2005). Aqueous two-phase partitioning is a potentially useful clarification or partial purification technique for a number of reasons: (1) it is a gentle method, having little or no adverse effect on the biological activity of most proteins; (2) many of the polymers used exhibit protein-stabilizing properties; (3) the yield of

protein recovered is generally high and (4) little technical difficulties arise during process scale-up (Walsh, 2001). ATPS has been applied to partitioning and recovery of various proteases such as trypsin (Oliveira *et al.*, 2002; Tubío *et al.*, 2007), α-chymotrypsin (Tubío *et al.*, 2007), chymosin, pepsin (Spelzini *et al.*, 2005), *Mucor bacilliformis* acid protease (Fernandez Lahore *et al.*, 1995), *Bacillus subtilis* neutral protease (Han and Lee, 1997) and tuna spleen proteinase (Klomklao *et al.*, 2005). Therefore, the objective of this study was to investigate the feasibility of utilizing ATPS for partitioning and recovery of protease from the stomach of albacore tuna.

4.3 Materials and Methods

4.3.1 Chemicals

Polyethylene glycol (PEG) 1000 was obtained from Wako Pure Chemical Industries, Ltd. (Tokyo, Japan). PEG2000, PEG4000, trichloroacetic acid, tris (hydroxymethyl) aminomethane and Folin-Ciocalteu's phenol reagent were obtained from Merck (Darmstadt, Germany). Hemoglobin, β-mercaptoethanol (βME), L-tyrosine, wide range molecular-weight markers and Coomassie Brilliant Blue G-250 were purchased from Sigma Chemical Co. (St. Louis, MO, USA.). Sodium dodecyl sulfate (SDS) and bovine serum albumin were obtained from Fluka (Buchs, Switzerland). *N,N,N',N'*-tetramethyl ethylene diamine (TEMED) was purchased from Bio-Rad Laboratories (Hercules, CA, USA). The salts and other chemicals with the analytical grade were procured from Merck (Darmstadt, Germany).

4.3.2 Fish stomach collection and preparation

Pooled internal organ of albacore tuna (*Thunnus alalunga*) was obtained from Tropical Canning (Thailand) Public Co., Ltd., Songkhla, Thailand. The sample was packed in polyethylene bag, kept in ice with a solid/ice ratio of 1:2 (w/v) and transported to the Department of Food Technology, Prince of Songkla University, Hat Yai within 1 h. Upon the arrival, internal organ was excised and only stomach

was collected and placed in polyethylene bag. The stomach sample was stored at -20 °C until use.

4.3.3 Preparation of stomach extract

Frozen stomach was thawed using running water (26-28 °C) until the core temperature reached -2 to 0 °C. The sample was cut into pieces with a thickness of 1-1.5 cm. Sample was rapidly frozen in liquid nitrogen and then finely ground using a National Model MX-T2GN blender (Taipei, Taiwan) to a powder form. Stomach powder was suspended in deionized water at a ratio of 1:9 (w/v). The mixture was stirred continuously at 4 °C for 30 min. The suspension was centrifuged for 30 min at 4 °C at 8,000 × *g* using a Sorvall Model RC-B Plus refrigerated centrifuge (Newtown, CT, USA) to remove the tissue debris. The supernatant was collected and referred to as "stomach extract (SE)". To activate stomach protease, SE was adjusted to pH 2 with 1 M HCl and the mixture was allowed to stand at 4 °C for 30 min (Zhou *et al.*, 2007). The suspension was centrifuged for 30 min at 4 °C at 10,000 × *g* using a refrigerated centrifuge. The collected supernatant was referred to as "acidified stomach extract (ASE)".

4.3.4 Enzyme assay

Protease activity of tuna stomach extract was determined using hemoglobin as a substrate according to the method of An *et al.* (1994) and Nalinanon *et al.* (2007) with a slight modification. To initiate the reaction, 200 µl of stomach extract was added into the assay mixture containing 200 µl of 2% hemoglobin, 200 µl of distilled water and 625 µl of reaction buffer. Appropriate dilution was made to ensure that the amount of enzyme was not excessive for available substrate in the assay system. The reaction was conducted at pH 2.0 and 50 °C for 20 min. To terminate enzymatic reaction, 200 µl of 50% (w/v) trichloroacetic acid (TCA) were added. Unhydrolyzed protein substrate was allowed to precipitate for 15 min at 4 °C, followed by centrifuging at 4,725 × *g* for 10 min using a MIKRO 20 centrifuge

(Hettich Zentrifugen, Tuttlingen, Germany). The oligopeptide content in the supernatant was determined by the Lowry method (Lowry *et al.*, 1951) using tyrosine as a standard. One unit of activity was defined as that releasing 1 μmole of tyrosine per min (μmol Tyr/min). A blank was run in the same manner, except that the stomach extract was added into the reaction mixture after the addition of 50% TCA (w/v). Protein concentration of stomach extract was measured by the method of Lowry *et al.* (1951) using bovine serum albumin as a standard. Specific activity was calculated and expressed as units/mg protein.

4.3.5 Preparation of aqueous two-phase systems

ATPS was prepared in 10-ml centrifuge tubes by adding the different amounts of PEG and salts together with SE or ASE according to the method of Klomklao *et al.* (2005).

4.3.5.1 Effect of salts on partitioning of protease in SE and ASE

To study the effect of salts on partitioning of the protease in SE and ASE using ATPS, different salts including $(NH_4)_2SO_4$, $Na_3C_6H_5O_7$, $MgSO_4$ and K_2HPO_4 at different concentrations (15, 20 and 25%, w/w) were mixed with 20% PEG1000 in aqueous system. One mL of SE or ASE (1 mg protein/mL) was added into the system. Distilled water was used to adjust the system to obtain the final weight of 5 g. The mixtures were mixed continuously for 3 min using a Vortex mixer (Vortex-genie2, G-560E, USA). Phase separation was achieved by centrifugation for 5 min at 2,000 × *g*. Top phase was carefully separated using a pasteur pipette and the interface of each tube was discarded. Volumes of the separated phases, top and bottom phases, were measured. Aliquots from each phase were taken for enzyme assay and protein determination.

The specific activity of stomach protease in the aqueous-two phase system was defined as:

$$SA = \frac{\text{Protease activity}}{\text{Protein concentration}}\text{; (unit/mg protein)} \qquad (1)$$

the purification factor as:

$$PF = \frac{SA_e}{SA_i} \quad (2)$$

where SA_e is the SA of each phase and SA_i is the initial SA of SE or ASE.

The partition coefficient of protein concentration was defined as:

$$K_P = \frac{C_T}{C_B} \quad (3)$$

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

The volume ratio as:

$$V_R = \frac{V_T}{V_B} \quad (4)$$

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

and the protease activity recovery yield as:

$$\text{Yield}\,(\%) = \frac{A_T}{A_i} \times 100 \quad (5)$$

where A_T is total protease activity in top phase and A_i is the initial protease activity of SE or ASE.

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

4.3.5.2. Effect of molecular weight and concentration of PEG on partitioning of protease in SE and ASE

To study the effect of the concentrations (15, 20 and 25%, w/w) of PEG1000, PEG2000 and PEG4000 on partitioning of protease in SE and ASE, 20% and 15% $MgSO_4$ were used in the system for SE and ASE partitioning, respectively. Partitioning was performed as described previously. Based on purity and recovery yield, the ATPS rendering the most effective partitioning was chosen for further study.

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

SDS-PAGE of SE, ASE and their selected ATPS fractions was performed according to the method of Laemmli (1970). Protein solutions were mixed

at 1:1 (v/v) ratio with the SDS-PAGE sample buffer (0.125 M Tris–HCl (pH 6.8), 4% SDS, 20% glycerol, 10% β-mercaptoethanol) and boiled for 3 min. The samples (0.75 μg protein) were loaded onto the gel made of 4% stacking and 12% separating gels and subjected to electrophoresis at a constant current of 15 mA per gel using a Mini-Protean II Cell apparatus (Bio-Rad Laboratories, Richmond, CA, USA). After electrophoresis, the gel was fixed with fixing solution, containing 40% (v/v) ethanol and 10% (v/v) acetic acid, for 60 min. The gel was then stained overnight with staining solution (0.1% (w/v) Coomassie Brilliant Blue G-250 in 2% (w/v) ortho-phosphoric acid and 10% (w/v) ammonium sulfate that prepared at least 24 h prior to mixing with methanol at a ratio of 4:1 (v/v)). Protein patterns were visualized after washing the gel with 1% (v/v) acetic acid for several times to remove Coomassie particles until clear background was observed.

4.3.7 Storage stability of stomach protease

Protease activity of SE and ASE and their selected ATPS fractions (PSE and PASE, respectively) containing 0.1% (w/v) sodium azide was monitored daily for the first 4 days and thereafter every 2 days up to 2 weeks. The storage temperature studied included 0 °C, 4 °C and room temperature (25-27 °C).

4.3.8 Protein determination

Protein concentration was measured by the Bradford method (Kruger, 1996) using bovine serum albumin as a standard.

4.3.9 Statistical analysis

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, 1.2, 1998) was used for data analysis.

4.4 Results and Discussion

4.4.1 Effect of salts on partitioning of stomach protease in ATPS

Partitioning of SE and ASE from albacore tuna was carried out in several biphasic systems of 20% PEG1000 containing different salts, including $(NH_4)_2SO_4$, $Na_3C_6H_5O_7$, $MgSO_4$ and K_2HPO_4 at various concentrations (Tables 9 and 10, respectively). After phase separation, two phases were obtained, PEG-rich top phase and salt-rich lower phase. However, no phase separation was observed in the system containing 15% $Na_3C_6H_5O_7$ and 15% $MgSO_4$ for partitioning of SE. For ASE partitioning, activity in both phases from the system containing K_2HPO_4 was not detectable, while varying activity was observed in other systems. The result suggested that acidified proteases in ASE were completely denatured under the alkaline pH of PEG/K_2HPO_4 system (pH 8.8). The phase volume ratio (V_R) of all systems tested was generally decreased when salt concentration increased. The addition of salts to the aqueous PEG solution led to an arrangement of ordered water molecules around PEG molecule due to their water structure breaking effects (Farruggia *et al.*, 2004). The formation of a water layer around the cation resulted in a more compact structure with a minor volume of PEG molecule.

Table 9. Effect of phase composition in PEG1000–Salt ATPS on partitioning of protease from the stomach of albacore tuna*

Phase composition (%, w/w)	V_R	K_P	SA	PF	Yield (%)
20% PEG1000–15% $(NH_4)_2SO_4$	1.16 ± 0.02^{i}**	1.45 ± 0.03^{d}	18.7 ± 0.40^{e}	4.18 ± 0.08^{e}	81.6 ± 0.61^{f}
20% PEG1000–20% $(NH_4)_2SO_4$	0.68 ± 0.01^{c}	1.37 ± 0.06^{d}	18.1 ± 0.85^{e}	4.06 ± 0.19^{e}	67.7 ± 0.08^{b}
20% PEG1000–25% $(NH_4)_2SO_4$	0.58 ± 0.01^{a}	1.21 ± 0.03^{c}	16.9 ± 0.76^{d}	3.79 ± 0.18^{d}	60.3 ± 0.79^{a}
20% PEG1000–15% $Na_3C_6H_5O_7$	ns	ns	ns	ns	ns
20% PEG1000–20% $Na_3C_6H_5O_7$	0.85 ± 0.01^{e}	0.81 ± 0.02^{a}	6.12 ± 0.14^{c}	1.37 ± 0.03^{c}	79.4 ± 0.93^{e}
20% PEG1000–25% $Na_3C_6H_5O_7$	0.75 ± 0.01^{d}	1.05 ± 0.09^{b}	5.17 ± 0.28^{b}	1.16 ± 0.06^{b}	76.8 ± 0.08^{d}
20% PEG1000–15% $MgSO_4$	ns	ns	ns	ns	ns
20% PEG1000–20% $MgSO_4$	1.26 ± 0.03^{h}	1.23 ± 0.02^{c}	27.7 ± 0.17^{g}	6.19 ± 0.04^{g}	87.9 ± 0.20^{g}
20% PEG1000–25% $MgSO_4$	0.93 ± 0.01^{f}	1.10 ± 0.04^{b}	19.9 ± 0.39^{f}	4.46 ± 0.09^{f}	73.3 ± 1.13^{c}
20% PEG1000–15% K_2HPO_4	1.00 ± 0.02^{g}	1.92 ± 0.04^{e}	4.35 ± 0.22^{a}	0.97 ± 0.05^{a}	80.2 ± 0.78^{e}
20% PEG1000–20% K_2HPO_4	0.65 ± 0.01^{c}	2.17 ± 0.07^{f}	4.30 ± 0.48^{a}	0.96 ± 0.11^{a}	60.8 ± 0.64^{a}
20% PEG1000–25% K_2HPO_4	0.61 ± 0.01^{b}	3.00 ± 0.10^{g}	4.10 ± 0.25^{a}	0.92 ± 0.06^{a}	60.6 ± 0.76^{a}

V_R: volume ratio (upper/lower); K_P: partition coefficient of protein in the upper phase; SA: specific activity (unit/mg protein); PF: purification factor; Yield: activity recovery yield. ns: no phase separation. *Means ± SD from triplicate determinations. ** Different superscripts in the same column indicate the significant differences ($P < 0.05$).

Table 10. Effect of phase composition in PEG1000–Salt ATPS on partitioning of acidified protease from the stomach of albacore tuna*

Phase composition (%, w/w)	V_R	K_P	SA	PF	Yield (%)
20% PEG1000–15% $(NH_4)_2SO_4$	0.98 ± 0.02^{h}**	1.74 ± 0.12^{d}	11.8 ± 0.49^{e}	1.63 ± 0.07^{e}	60.5 ± 1.49^{e}
20% PEG1000–20% $(NH_4)_2SO_4$	0.67 ± 0.01^{c}	1.96 ± 0.25^{de}	9.88 ± 0.12^{c}	1.36 ± 0.02^{c}	53.1 ± 0.47^{c}
20% PEG1000–25% $(NH_4)_2SO_4$	0.44 ± 0.02^{a}	1.12 ± 0.07^{bc}	11.8 ± 0.44^{e}	1.63 ± 0.06^{e}	38.0 ± 0.50^{a}
20% PEG1000–15% $Na_3C_6H_5O_7$	1.37 ± 0.01^{j}	0.95 ± 0.03^{ab}	10.5 ± 0.15^{d}	1.45 ± 0.02^{d}	75.7 ± 0.39^{g}
20% PEG1000–20% $Na_3C_6H_5O_7$	0.89 ± 0.03^{e}	0.77 ± 0.01^{a}	8.69 ± 0.03^{b}	1.20 ± 0.01^{b}	60.0 ± 0.19^{de}
20% PEG1000–25% $Na_3C_6H_5O_7$	0.73 ± 0.01^{d}	0.76 ± 0.03^{a}	6.41 ± 0.05^{a}	0.88 ± 0.01^{a}	46.7 ± 0.42^{b}
20% PEG1000–15% $MgSO_4$	2.52 ± 0.02^{k}	2.21 ± 0.09^{e}	15.9 ± 0.42^{f}	2.19 ± 0.01^{f}	81.1 ± 0.88^{h}
20% PEG1000–20% $MgSO_4$	1.16 ± 0.01^{i}	2.05 ± 0.29^{e}	16.0 ± 0.16^{f}	2.20 ± 0.05^{f}	62.2 ± 0.21^{f}
20% PEG1000–25% $MgSO_4$	0.95 ± 0.02^{g}	1.78 ± 0.27^{d}	16.0 ± 0.06^{f}	2.21 ± 0.01^{f}	59.0 ± 0.13^{d}
20% PEG1000–15% K_2HPO_4	0.91 ± 0.01^{f}	1.73 ± 0.12^{d}	na	na	-
20% PEG1000–20% K_2HPO_4	0.72 ± 0.01^{d}	1.33 ± 0.01^{c}	na	na	-
20% PEG1000–25% K_2HPO_4	0.59 ± 0.01^{b}	0.84 ± 0.04^{a}	na	na	-

V_R: volume ratio (upper/lower); K_P: partition coefficient of protein in the upper phase; SA: specific activity (unit/mg protein); PF: purification factor; Yield: activity recovery yield. na: no protease activity. *Means ± SD from triplicate determinations. ** Different superscripts in the same column indicate the significant differences ($P < 0.05$).

For all ATPS studied, the proteases in both SE and ASE were partitioned predominantly in the PEG-rich top phase as evidenced by negligible or no protease activity in bottom phase (data not shown). In general, negatively charged proteins prefer the upper phase in PEG-salt systems, while positively charged proteins normally partition selectively to the bottom phase (del-Val and Otero, 2003; Gautam and Simon, 2006; Klomklao *et al.*, 2005; Yang *et al.*, 2008). Above the isoelectric point (p*I*), enzyme/protein polyanions are accepted by PEG-rich phase in ATPS (Huddleston *et al.*, 1991; Tanuja *et al.*, 1997). This contrasts with the rejection of inorganic polyanions and may be attributed to an inherent greater separation of point charges on amino acid side-chains over the surface of enzyme polyanions (Tanuja *et al.*, 1997). Such separation enhances the ability of the enzyme to interact with the PEG (Tanuja *et al.*, 1997). Reh *et al.* (2007) reported that the transfer of both proteins, trypsin and α-chymotrypsin to top phase was associated with negative enthalpic and entropic changes. Hence, stomach proteases, both non-activated and activated, partitioned in the top phase might be negatively charged. These results might be explained by acceptance of enzyme polyanions by PEG-rich phase in ATPS that occured above p*I*. Since pepsin, a major acidic protease in stomach has p*I* less than 1 (Whitaker, 1994), the enzyme was negatively charged in all partitioning systems, which had pH ranging from 4.6 to 8.8. Xia *et al.* (1993) studied the PEG-pepsin interaction at acidic pHs (1-4), postulating an electrostatic interaction between the protonated carboxyl groups of pepsin and the oxygen ether of PEG by hydrogen bond formation. Therefore, stomach proteases from albacore tuna might be partitioned towards the PEG-rich phase in all systems due to electrostatic interaction between enzyme surface and PEG. In PEG-salt systems, anions and cations distribute themselves differently between the phases depending on their affinity with water. Generally, two phases have to be electrically neutral and iso-osmotic. Thus, an interfacial potential is produced differently which may aid the partitioning of charged biomolecules (del-Val and Otero, 2003). SA, PF and activity recovery (% yield) of protease obtained from PEG1000-salt systems depended on types of salt used. The phase system containing $MgSO_4$ showed superior partitioning affinity for both SE and ASE to those containing other salts. For SE, a phase system containing 20% PEG1000 and 20% $MgSO_4$ gave the highest SA (27.7 units/mg protein), PF (6.2-fold) and yield

(87.9%) ($P < 0.05$). The maximal yield of 81.1% from the system containing 20% PEG1000 and 15% $MgSO_4$ was obtained for partitioning of ASE ($P < 0.05$). Similar SA and PF were observed in system with other $MgSO_4$ concentrations ($P > 0.05$). Therefore, the systems containing 20% or 15% $MgSO_4$ were appropriate for partitioning of protease in SE and ASE, respectively.

Partition coefficient (K) is used to characterize the proteins distribution in ATPS. K values for protease and protein partitioning are reported as K_E and K_P, respectively. Since protease was found only in upper phase, K_E of SE and ASE could not be calculated. Generally, the lowest K_P indicates a shift of contaminant proteins, nucleic acid and other undesirable components to the lower phase. When a single component must be extracted from a mixture, phase system compositions are often manipulated in such a manner that the component partitions into one of the phases, while the other components of the mixture partition into the other phase (Reh *et al.*, 2002). K_P values varied with types and concentrations of salt used for SE partitioning. Increasing salts concentration for ASE partitioning resulted in lower K_P. Salts might enhance the migration of the proteins to the lower phase by electrostatic repulsion effects (Han and Lee, 1997). Gündüz and Korkmaz (2000) observed that an increase in phosphate concentration caused a decrease in the partition coefficient of negatively charged BSA at pH 7 (pI = 4.82). Therefore, the distribution of protein in ATPS of SE and ASE likely depended on the differences in surface charge of protease and others protein contaminants, which were governed by different salts. Han and Lee (1997) mentioned that the surface charge of biological materials is one of the most significant factors affecting the separation of partitioning system. Furthermore, molecular weight, shape, hydrophobicity and specific binding sites of biological materials also affect the partition profiles. Electrical interaction and repulsion between charged aqueous phase systems and the proteins affect the partitioning of system (Han and Lee, 1997). Tables 9 and 10 show that increasing salt concentration resulted in a less activity recovery. This might be due to the denaturation of proteases causes by "salting out effect". The same result was reported on partitioning of proteinase from tuna spleen (Klomklao *et al.*, 2005). Antov *et al.* (2006) found that increasing in concentration of ammonium sulphate resulted in less recovery yield of xylanase in the top phase.

4.4.2 Effect of PEG molecular weight on partitioning of stomach protease in ATPS

Partitioning of protease in SE and ASE using ATPS containing PEG with varying molecular weights and concentrations in the presence of 20% $MgSO_4$ or 15% $MgSO_4$ for SE and ASE, respectively, are shown in Tables 11 and 12. The results showed that all partitioning parameters in PEG/$MgSO_4$ systems were influenced by PEG molecular weight. At the same PEG concentration, K_P decreased with increasing PEG molecular weight. A preferential interaction between PEG molecule and protein domain decreased when the molecular mass of PEG increased due to its exclusion from the protein domain (Klomklao *et al.*, 2005; Reh *et al.*, 2002). Tubío *et al.* (2007) suggested that for ATPS formed by PEG of low molecular weight (600-3350 kDa), the protein transfer to the top phase is enthalpically driven mainly due to a strong interaction between PEG and the protein (Tubío *et al.*, 2007). PEG of the highest molecular weight (PEG8000) exclude the protein from the top phase driven by an entropically unfavorable term (Tubío *et al.*, 2007). For systems formed by PEG of low molecular weight, the free energy changes adopt negative values (enthalpic changes overcomes entropic changes), while opposite behaviour ($\Delta G_{transfer}>0$) occurs from ATPSs formed by PEGs of higher molecular weights (Tubío *et al.*, 2007). Furthermore, better partitioning of protein was achieved with lower molecular weight PEG, compared to that with higher molecular weight possibly due to the fact that interfacial tension is lower when molecular weight of PEG is lower (Saravanan *et al.*, 2007). Therefore, PEG1000 was a suitable polymer for partitioning of protease in SE as indicated by the higher SA and PF than PEG with higher molecular weights. For partitioning of protease in ASE, PEG1000 showed high SA and PF. However, PEG2000 exhibited the higher activity recovery. It was speculated that ATPS system for protease purification might be governed by the state of enzyme, inactive (SE) or active (ASE), which possessed different physiochemical properties. ATPS of 25% PEG1000-20% $MgSO_4$ gave the highest PF (7.2-fold) with 85.7% yield for partitioning of protease in SE ($P < 0.05$). The 15% PEG2000-15% $MgSO_4$ afforded the greatest PF (2.4-fold) with 89.1% yield for ATPS in partitioning protease in ASE ($P < 0.05$). Those phase systems exhibiting the highest PF with

appropriate recovery yield for SE or ASE were selected for further study and those fractions were referred to “PSE” and “PASE”, respectively.

Table 11. Effect of PEG molecular mass and concentration in a PEG-$MgSO_4$ ATPS on partitioning of protease from the stomach of albacore tuna*

Phase composition (%, w/w)	V_R	K_P	SA	PF	Yield (%)
15% PEG1000–20% $MgSO_4$	1.02 ± 0.01^{c}**	0.62 ± 0.02^{e}	22.7 ± 0.63^{de}	6.11 ± 0.07^{d}	84.2 ± 0.71^{e}
20% PEG1000–20% $MgSO_4$	1.25 ± 0.01^{e}	1.24 ± 0.03^{f}	23.5 ± 0.91^{e}	6.34 ± 0.24^{e}	87.5 ± 0.52^{fg}
25% PEG1000–20% $MgSO_4$	1.61 ± 0.01^{h}	1.30 ± 0.05^{f}	26.8 ± 0.60^{f}	7.23 ± 0.16^{f}	85.7 ± 0.56^{ef}
30% PEG1000–20% $MgSO_4$	1.71 ± 0.01^{j}	1.61 ± 0.09^{g}	22.0 ± 0.97^{d}	5.92 ± 0.26^{d}	76.0 ± 1.37^{a}
15% PEG2000–20% $MgSO_4$	0.78 ± 0.02^{b}	0.26 ± 0.01^{ab}	11.5 ± 0.42^{b}	3.10 ± 0.11^{b}	91.9 ± 1.41^{hi}
20% PEG2000–20% $MgSO_4$	1.07 ± 0.01^{d}	0.35 ± 0.02^{cd}	11.4 ± 0.23^{b}	3.06 ± 0.06^{b}	98.6 ± 1.24^{k}
25% PEG2000–20% $MgSO_4$	1.55 ± 0.01^{g}	0.36 ± 0.01^{d}	12.9 ± 0.26^{c}	3.49 ± 0.07^{c}	94.6 ± 0.34^{j}
30% PEG2000–20% $MgSO_4$	1.74 ± 0.01^{k}	0.60 ± 0.05^{e}	3.26 ± 0.32^{a}	0.88 ± 0.09^{a}	33.3 ± 0.24^{b}
15% PEG4000–20% $MgSO_4$	0.75 ± 0.01^{a}	0.25 ± 0.01^{a}	12.7 ± 0.20^{c}	3.41 ± 0.05^{c}	93.2 ± 0.24^{ij}
20% PEG4000–20% $MgSO_4$	1.07 ± 0.01^{d}	0.28 ± 0.02^{abc}	13.2 ± 0.12^{c}	3.55 ± 0.03^{c}	89.9 ± 0.77^{gh}
25% PEG4000–20% $MgSO_4$	1.47 ± 0.01^{f}	0.34 ± 0.05^{bcd}	3.32 ± 0.31^{a}	0.89 ± 0.08^{a}	50.6 ± 1.43^{c}
30% PEG4000–20% $MgSO_4$	1.66 ± 0.01^{i}	0.38 ± 0.09^{d}	3.30 ± 0.43^{a}	0.89 ± 0.11^{a}	22.9 ± 3.95^{a}

V_R: volume ratio (upper/lower); K_P: partition coefficient of protein in the upper phase; SA: specific activity (unit/mg protein); PF: purification factor; Yield: activity recovery yield. *Means ± SD from triplicate determinations. ** Different superscripts in the same column indicate the significant differences ($P < 0.05$).

Table 12. Effect of PEG molecular mass and concentration in a PEG-$MgSO_4$ ATPS on partitioning of acidified protease from the stomach of albacore tuna*

Phase composition (%, w/w)	V_R	K_P	SA	PF	Yield (%)
15% PEG1000–15% $MgSO_4$	ns	ns	ns	ns	ns
20% PEG1000–15% $MgSO_4$	2.49 ± 0.01^{h}**	2.18 ± 0.07^{f}	15.5 ± 0.98^{f}	2.22 ± 0.14^{f}	79.2 ± 2.31^{b}
25% PEG1000–15% $MgSO_4$	2.78 ± 0.02^{j}	2.24 ± 0.05^{fg}	14.2 ± 0.11^{e}	2.03 ± 0.02^{e}	79.3 ± 0.13^{b}
30% PEG1000–15% $MgSO_4$	2.92 ± 0.01^{k}	2.33 ± 0.03^{g}	11.2 ± 0.55^{d}	1.60 ± 0.08^{d}	74.7 ± 0.35^{a}
15% PEG2000–15% $MgSO_4$	1.60 ± 0.01^{c}	0.86 ± 0.07^{a}	17.0 ± 0.69^{g}	2.43 ± 0.10^{g}	89.1 ± 3.03^{e}
20% PEG2000–15% $MgSO_4$	1.86 ± 0.02^{d}	1.34 ± 0.05^{c}	15.8 ± 0.03^{f}	2.26 ± 0.01^{f}	96.3 ± 1.24^{f}
25% PEG2000–15% $MgSO_4$	2.23 ± 0.01^{f}	2.04 ± 0.04^{e}	10.9 ± 0.23^{d}	1.56 ± 0.03^{d}	97.9 ± 1.01^{f}
30% PEG2000–15% $MgSO_4$	2.52 ± 0.01^{i}	2.33 ± 0.04^{g}	8.46 ± 0.13^{b}	1.21 ± 0.02^{b}	86.3 ± 1.03^{d}
15% PEG4000–15% $MgSO_4$	1.17 ± 0.01^{a}	1.11 ± 0.04^{b}	9.89 ± 0.13^{c}	1.42 ± 0.02^{c}	82.1 ± 1.19^{c}
20% PEG4000–15% $MgSO_4$	1.55 ± 0.01^{b}	1.40 ± 0.04^{c}	10.1 ± 0.17^{c}	1.45 ± 0.02^{c}	87.7 ± 0.48^{de}
25% PEG4000–15% $MgSO_4$	1.95 ± 0.01^{e}	1.91 ± 0.09^{d}	9.45 ± 0.11^{c}	1.35 ± 0.02^{c}	96.0 ± 0.76^{f}
30% PEG4000–15% $MgSO_4$	2.45 ± 0.01^{g}	2.16 ± 0.03^{f}	7.19 ± 0.11^{a}	1.03 ± 0.02^{a}	96.8 ± 0.46^{f}

V_R: volume ratio (upper/lower); K_P: partition coefficient of protein in the upper phase; SA: specific activity (unit/mg protein); PF: purification factor; Yield: activity recovery yield. ns: no phase separation. *Means ± SD from triplicate determinations. ** Different superscripts in the same column indicate the significant differences ($P < 0.05$).

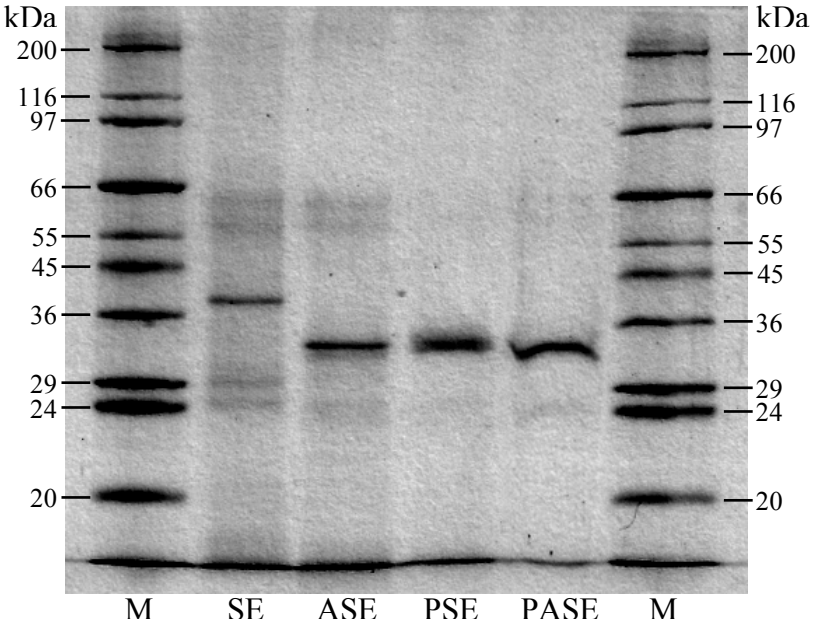

Figure 15. SDS–PAGE patterns of stomach extract (SE) and acidified stomach extract (ASE), and their ATPS fractions (PSE and PASE). M denotes wide range MW protein markers.

4.4.3 Protein pattern of stomach protease partitioned with ATPS

The purity of protease from the stomach of albacore tuna after ATPS process was analyzed by SDS-PAGE (Figure 15). SE contained a major band with a molecular weight (MW) of 40.6 kDa. Proteins with MWs of 25, 30, 56 and 62 kDa were also found. After acidification process, some of protein contaminants were removed and the major band with MW of 32.7 kDa was observed. Additionally, a large number of contaminating proteins were also removed after ATPS partitioning. A greater intensity of major protein band (32.7 kDa) was obtained in both PSE and PASE, in comparison with that found in SE and ASE. The major protease in stomach extract of albacore tuna has been reported to be aspartic proteinase, most likely pepsin (Nalinanon *et al.*, 2008b). Fish pepsin had MW of 30 kDa and 35 kDa for sea bream (Zhou *et al.*, 2007) and smooth hound (Bougatef *et al.*, 2008). The migration of major protein band of SE to lower molecular weight position after acidification or ATPS partitioning might be due to the autocatalytic activation of pepsinogen to pepsin under acidic condition and with two-phase systems, respectively. This finally removed 44 amino acid residues (the prosegment) from the N-terminal end of the zymogen peptide chain (Nielsen and Foltmann, 1993). Nalinanon *et al.* (2008b) found that the activation of pepsin from tuna stomach, including albacore tuna, skipjack tuna and tongol tuna could be achieved at pH below 5.

4.4.4 Storage stability of stomach protease partitioned with ATPS

Figure 16 shows the changes in protease activity of SE, ASE, PSE and PASE during storage at different temperatures up to 14 days. At 0 °C and 4 °C, SE was quite stable throughout 14 days of storage. However, the stability of PSE was lower as indicated by continuous decrease in activity with increasing storage time. Protease activity of ASE and PASE gradually decreased when storage time increased (Figure 16A and 16B). Nevertheless, the greatest loss in protease activity was observed in PASE when compared to ASE and PSE. The greater decrease in protease activity was observed when stored at room temperature. For ASE, PSE and PASE, more than 80% of the initial activity was lost at day 3 of storage at room temperature

(Figure 16C). It was noted that the stability of stomach protease most likely depended on the form of enzyme and storage temperature. Additionally, PEG present in PSE and PASE had no stabilizing effect on protease. Protein-stabilizing ability of PEG has been reported (Lee and Lee, 1981; Reh *et al.*, 2007). Instability of stomach protease might be mainly due to autolysis process.

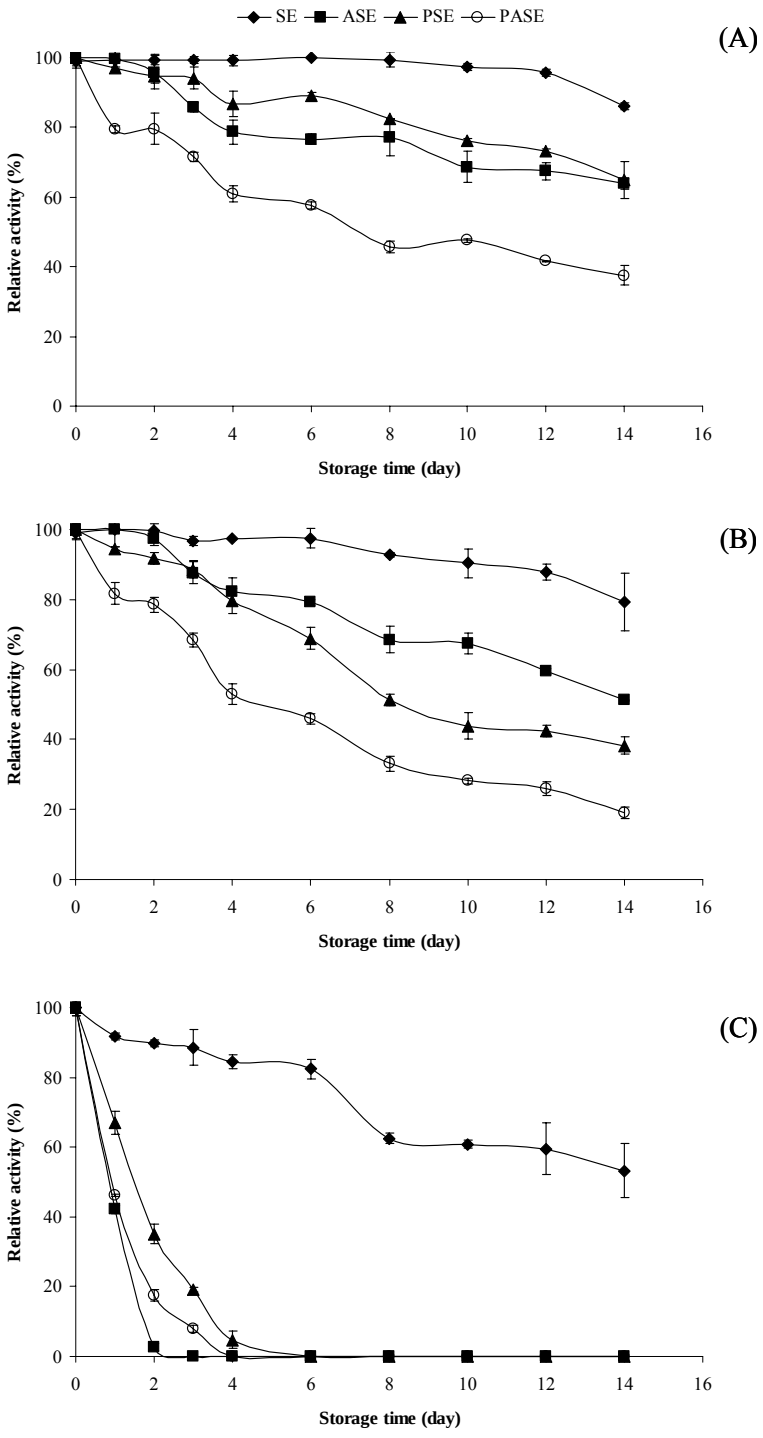

Figure 16. Changes in protease activity of stomach extract (SE) and acidified stomach extract (ASE), and their ATPS fractions (PSE and PASE) during storage at 0 °C (A), 4 °C (B) and room temperature (C). Bars represent standard deviation from triplicate determinations.

4.5 Conclusion

The optimum condition for partitioning of SE and ASE from the stomach of albacore tuna was 25% PEG1000-20% $MgSO_4$ and 15% PEG2000-15% $MgSO_4$, respectively. The highest PF of 7.2-fold and 2.4-fold, with the yield of 85.7% and 89.1% was obtained for SE and ASE, respectively. After phase separation, protein with MW of 32.7 kDa became dominant. Protease in SE was stable at 0 °C and 4 °C throughout 14 days of storage, while ASE, PSE and PASE gradually lost their activity in time-dependent manner. Instability of protease was more pronounced in higher storage temperature. ATPS can be effectively used to recover and purify protease from albacore tuna stomach. However, changes in protease properties after partitioning should be considered.

CHAPTER 5

TUNA PEPSIN: CHARACTERISTICS AND ITS USE FOR COLLAGEN EXTRACTION FROM THE SKIN OF THREADFIN BREAM (*NEMIPTERUS SPP.*)

5.1 Abstract

Pepsin from the stomach of albacore tuna, skipjack tuna, and tongol tuna was characterized. Pepsin from all tuna species showed maximal activity at pH 2.0 and 50 °C, when hemoglobin was used as a substrate. Among the stomach extract of all species tested, that of albacore tuna showed the highest activity (40.55 units/g tissue) ($P < 0.05$). Substrate-Native-PAGE revealed that pepsin from albacore tuna and tongol tuna consisted of 2 isoforms, whereas pepsin from skipjack tuna had only 1 form. The activity was completely inhibited by pepstatin A, while EDTA (ethylenediaminetetraacetic acid), SBTI (soybean trypsin inhibitor), and E-64 (1-(*L*-trans-epoxysuccinyl-leucylamino)-4-guanidinobutane) exhibited negligible effect. The activity was strongly inhibited by SDS (sodium dodecyl sulfate) (0.05-0.1% (w/v)). Cysteine (5-50 mM) also showed the inhibitory effect in a concentration dependent manner. ATP, molybdate, NaCl, $MgCl_2$, and $CaCl_2$ had no impact on the activity. When tuna pepsin (10 units/g defatted skin) was used for collagen extraction from the skin of threadfin bream for 12 h, the yield of collagen increased by 1.84-2.32 folds and albacore pepsin showed the comparable extraction efficacy to porcine pepsin. The yield generally increased with increasing extraction time ($P < 0.05$). All collagen obtained with the aid of tuna pepsin showed similar protein patterns, compared with those found in acid-solubilzed collagen. Nevertheless, pepsin from skipjack tuna caused the degradation of α and β components. All collagens were classified as type I with large portion of β-chain. However, proteins with MW greater than 200 kDa were abundant in acid-solubilized collagen.

5.2 Introduction

Thailand is the top canned tuna producer and exporter in the world (Department of Foreign Trade, 2007). By the year 2006, Thai canned tuna was exported to several countries with the total volume of 416,226 metric tons and a value of 40,408 million Bahts (The Customs Department, 2007). Several tuna species, including albacore, skipjack, tongol, and yellowfin, are mainly used for the production of canned tuna in Thailand. During canned tuna manufacturing, high amount of processing wastes, such as viscera, head, and bone are discarded. Among those byproducts, fish viscera can be used as a potential source of various enzymes, particularly proteases (Klomklao *et al.*, 2004). Pepsin is the major digestive enzyme in stomach of animals that are secreted as pepsinogen from chief cells of oxyntic glands located in the stomach wall epithelium. In acidic environment, pepsinogen rapidly converts to pepsin (Kageyama, 2002). Pepsins and pepsinogens from stomach of various fish species such as Arctic fish capelin (Gildberg and Raa, 1983), polar cod (Arunchalam and Haard, 1985), North Pacific bluefin tuna (Tanji *et al.*, 1988), Atlantic cod (Gildberg *et al.*, 1990), sea bream (Zhou *et al.*, 2007), African coelacanth (Tanji *et al.*, 2007), and pectoral rattail (Klomklao *et al.*, 2007) have been purified and characterized.

Apart from tuna industry, surimi industry has become important due to the increasing global market demand. Threadfin bream (*Nemipterus spp.*) is one of important raw materials used for surimi production in Thailand in addition to bigeye snapper, lizard fish, croacker, and others (Benjakul *et al.*, 2005). During surimi production, a large quantity of byproducts, especially skin and bone, are generated. Fish skin can serve as an alternative source for collagen since mammalian collagens are associated with several problems such as the outbreak of mad cow disease and the constraint for some religions, mainly Islam and Judaism. Therefore, skin collagen from several fish species, such as sheephead seabream (Ogawa *et al.*, 2003), Pacific whiting (Kim and Park, 2004), Nile perch (Muyonga *et al.*, 2004), channel catfish (Liu *et al.*, 2007), and bigeye snapper (Nalinanon *et al.*, 2007) has been extracted and characterized. Generally, typical process, especially acid solubilization, renders a low yield of collagen. To tackle the problem, pepsin has been applied since it is able to

cleave peptides specifically in telopeptide region of collagen, leading to the increased extraction efficiency (Nagai *et al.*, 2002; Nalinanon *et al.*, 2007). Use of commercial pepsin can be costly for collagen extraction. Tuna pepsin from stomach, a byproduct, can be a promising enzyme due to its abundance and unique properties. Therefore, the objectives of this study were to characterize pepsin from the stomach of different tuna species, including albacore, skipjack, and tongol tuna as well as to use tuna pepsin for collagen extraction from the skin of threadfin bream.

5.3 Materials and Methods

5.3.1 Chemicals

Bovine hemoglobin, *β*-mercaptoethanol (βME), *L*-tyrosine, *L*-cysteine, Adenosine 5′-triphosphate (ATP), molybdate, bovine serum albumin, pepsin from porcine stomach mucosa (EC 3.4.23.1; powdered; 750 U mg^{-1} dry matter), and wide range protein markers were purchased from Sigma Chemical Co. (St. Louis, MO, USA.). Trichloroacetic acid, disodium hydrogen phosphate, sodium citrate, Folin-Ciocalteu's phenol reagent, acetic acid, and *p*-dimethylamino-benzaldehyde were obtained from Merck (Darmstadt, Germany). Sodium dodecyl sulfate (SDS), Coomassie Blue R-250, and *N,N,N′,N′*-tetramethyl ethylene diamine (TEMED) were procured from Bio-Rad Laboratories (Hercules, CA, USA). Type I collagen from calf skin was purchased from Elastin Products Co., Inc. (Owensville, MO, USA).

5.3.2 Preparation of fish stomach and skin

Internal organs of skipjack tuna (*Katsuwonus pelamis*), albacore tuna (*Thunnus alalunga*), and tongol tuna (*Thunnus tonggol*) were obtained from Tropical Canning (Thailand) Public Co., Ltd., Songkhla, Thailand. Those samples were packed in polyethylene bag, kept in ice with a solid/ice ratio of 1:2 (w/v) and transported to the Department of Food Technology, Prince of Songkla University, Hat Yai within 1 h. Upon the arrival, pooled internal organs from each species were excised and only

stomach was collected and placed in polyethylene bag. The stomach samples were stored at -20 °C until use.

Skins of threadfin bream (*Nemipterus spp.*) obtained from Man A Frozen Foods Co. Ltd., Songkhla, Thailand, were kept in ice and transported to the Department of Food Technology within 1 h. The residual meat was removed manually from the skins. The cleaned skin was then washed with tap water, drained and cut into small pieces (0.5 × 0.5 cm). The prepared skins were placed in polyethylene bag and stored at -20 °C until use.

5.3.3 Preparation of stomach extract

Frozen stomach was thawed using running water (26-28 °C) until the core temperature reached -2 to 0 °C. The sample was cut into pieces with a thickness of 1-1.5 cm. Sample was rapidly frozen in liquid nitrogen and then finely ground using a National Model MX-T2GN blender (Taipei, Taiwan) to a powder form. Stomach powder was suspended in 50 mM sodium phosphate buffer, pH 7.2, at a ratio of 1:9 (w/v) according to the method of Nalinanon *et al.* (2007). The mixture was stirred continuously at 4 °C for 30 min. The suspension was centrifuged for 30 min at 4 °C at 7,700 × *g* using a Sorvall Model RC-B Plus refrigerated centrifuge (Newtown, CT, USA) to remove the tissue debris. The supernatant was collected and referred to as “stomach extract”. The protein content in the extract was measured according to the method of Lowry *et al.* (1951).

5.3.4 Assay of proteolytic activity

Proteolytic activity of tuna stomach extract was determined using hemoglobin as a substrate according to the method of An *et al.* (1994) and Nalinanon *et al.* (2007) with a slight modification. To initiate the reaction, 200 µl of stomach extract was added into the assay mixture containing 200 µl of 2% hemoglobin, 200 µl of distilled water and 625 µl of reaction buffer. Appropriate dilution was made to ensure that the amount of enzyme was not excessive for available substrate in the

assay system. The reaction was conducted at pH 2.0 and 50 °C for 20 min. To terminate enzymatic reaction, 200 µl of 50% (w/v) trichloroacetic acid (TCA) were added. Unhydrolyzed protein substrate was allowed to precipitate for 15 min at 4 °C, followed by centrifuging at 4,725 × *g* for 10 min using a MIKRO 20 centrifuge (Hettich Zentrifugen, Tuttlingen, Germany). The oligopeptide content in the supernatant was determined by the Lowry method (Lowry *et al.*, 1951) using tyrosine as a standard (An *et al.*, 1994). One unit of activity was defined as that releasing 1 µmole of tyrosine per min (µmol Tyr/min). A blank was run in the same manner, except that the stomach extract was added into the reaction mixture after the addition of 50% TCA (w/v). Protein concentration of stomach extract was measured by the method of Lowry *et al.* (1951) using bovine serum albumin as a standard. Specific activity was calculated and expressed as units/mg protein.

5.3.5 pH and temperature profile

To study pH profile, proteolytic activity of stomach extract was measured at 50 °C using hemoglobin as a substrate at different pHs (pHs 1 and 1.5 using 20 mM maleate buffer and pHs 2.0, 2.5, 3.0, 3.5, 4.0, 5.0, 6.0 and 7.0 using McIlvaine's buffer (0.2 M Na-phosphate and 0.1 M Na-citrate)). For temperature profile, the assay was performed at various temperatures (25, 30, 35, 40, 45, 50, 55, 60, 65 and 70 °C) at pH 2.

5.3.6 Effect of some protease inhibitors

Stomach extract (200 µl) was mixed with 200 µl of single protease inhibitor to obtain the final designated concentration (0.1 mM E-64, 0.1 mM soybean trypsin inhibitor, 1 and 10 µM pepstatin A and 2 mM EDTA). Mixtures were incubated at room temperature (26-28 °C) for 20 min. The residual activity was measured at pH 2 and 50 °C. Relative activity was then calculated.

5.3.7 Effect of some chemicals

Different chemicals (200 µl) were mixed with stomach extract (200 µl) to obtain the concentration designated (0.1 and 0.5 mM for ATP and molybdate; 1 and 5 mM for NaCl, $MgCl_2$ and $CaCl_2$; 0.05 and 0.1% (w/v) for SDS, and 5, 25 and 50 mM for *L*-cysteine). The mixtures were kept at room temperature for 30 min. The remaining activity was determined at pH 2 and 50 °C and the relative activity was calculated as described previously.

5.3.8 Activity staining of tuna pepsin

Activity staining of tuna pepsin was carried out as per the method of Díaz-López *et al.* (1998) with a slight modification. Stomach extract was mixed with sample buffer containing no SDS and βME at a ratio of 1:1 (v/v). The samples (15 µg protein) were loaded onto the gels, consisting of 4% of stacking gel and 12% separating gel, where SDS was excluded. Electrophoresis was performed at constant current of 15 mA per gel at 4 °C. After electrophoresis, gels were removed and soaked in 0.1 M HCl to reduce the pH to 2.0 to activate the pepsin. After 15 min, the gels were soaked in hemoglobin solution (0.25% hemoglobin in 0.1 M glycine-HCl at pH 2.0) with constant agitation for 30 min at 4 °C to allow the substrate to penetrate into the gels. The gels were then soaked for 90 min in a fresh hemoglobin solution at 50 °C to develop the activity zone. After development of activity zone, gels were washed using distilled water and fixed for 15 min in 12% TCA solution prior to staining with 0.125% Coomassie blue R-250 in 45% methanol and 10% acetic acid and destained in 30% methanol and 10% acetic acid. Development of clear zones on blue background indicated proteolytic activity.

5.3.9 Use of tuna pepsin for the extraction of collagen from the skin of threadfin bream

Prior to collagen extraction, stomach extracts from different tuna species were adjusted to pH 2 with 1 M HCl and the mixture was allowed to stand at 4 °C for 10 min. The treated stomach extracts were centrifuged for 30 min at 4 °C at 5,000 × *g* using a refrigerated centrifuge. The acidified supernatants were collected and used for collagen extraction.

The collagens from the skin of threadfin bream were extracted following the method of Nalinanon *et al.* (2007) with a slight modification. All procedures were performed at 4 °C. To remove non-collagenous proteins, the skin was mixed with 0.1 N NaOH using a sample/alkaline solution ratio of 1:10 (w/v). The mixture was stirred continuously for 6 h. The alkaline solution was changed every 2 h. Then, the alkaline treated skin was washed with cold water until neutral or faintly basic pHs of wash water were obtained. The treated skin was then defatted 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 3 times.

To extract the collagen, prepared skin was soaked in 0.5 M acetic acid with a solid/solvent ratio of 1:15 (w/v) in the absence and presence of acidified tuna stomach extract with the activity of 10 units/g defatted skin. Porcine pepsin at the same level was also used for the comparative study. The mixture was stirred at 4 °C for 6 and 12 h. At the designated time, the mixture was immediately mixed with pepstatin A to obtain a final concentration of 1 μM. The mixture was filtered with 2 layers of cheesecloth to remove unhydrolyzed tissues. The filtrates were subjected to the determination of hydroxyproline content and SDS-PAGE as described by Nalinanon *et al.* (2007). The yield of collagen extracted was calculated based on hydroxyproline content in the extract in comparison with that of defatted skin.

5.3.10 Statistical analysis

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, 1.2, 1998) was used for data analysis.

5.4 Results and Discussion

5.4.1 pH and temperature profile of tuna pepsin

The effects of pH on the activity of pepsin from the stomach of albacore tuna, skipjack tuna, and tongol tuna are shown in Figure 17. The optimal pH for hydrolysis of hemoglobin of those pepsins was found at pH 2.0, which was in accordance with those of pepsins obtained from stomach of polar cod (Arunchalam and Haard, 1985), African coelacanth (Tanji *et al.*, 2007) and smooth hound (Bougatef *et al.*, 2008) when hemoglobin was used as a substrate. However, pepsins from North Pacific bluefin tuna (Tanji *et al.*, 1988) showed the maximal activity toward hemoglobin at pH 2.5. Bohak (1973) found that the conversion of pepsinogen to pepsin was observed in pH range of 2-4. In this pH range, the zymogen undergoes a rapid conformational change to form an intermediate, and the subsequent conversion of this intermediate to the active enzyme. At pH 2, stomach extract from albacore tuna exhibited the highest specific activity, followed by pepsin from skipjack tuna and tongol tuna, respectively. The activities of tuna pepsin decreased generally at very acidic pH and sharply decreased as the pH was above 3.0. At pHs higher and lower than the optimal pH, the enzyme possibly underwent the conformational changes under harsh condition, resulting in the lowered activity (Benjakul *et al.*, 2003). It was noted that no activity was observed at pH 5 or above. Therefore, the activation of tuna pepsin could be achieved at pH below 5.

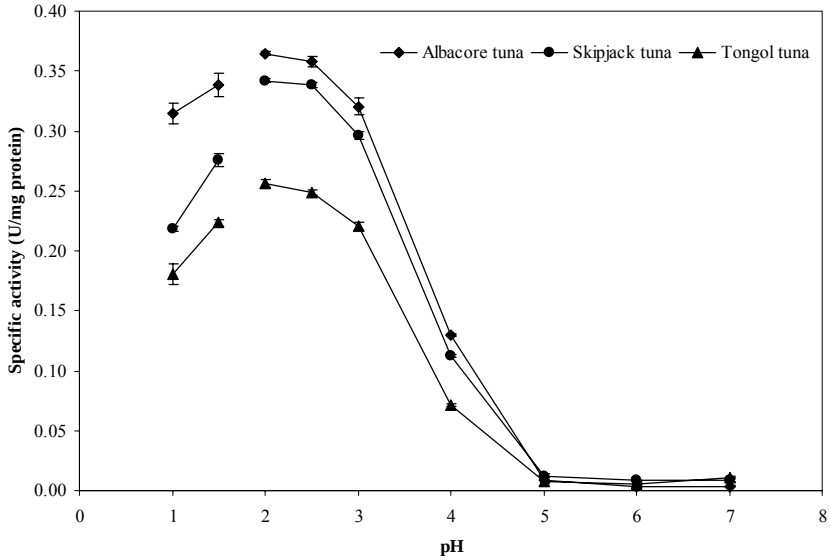

Figure 17. pH profiles of pepsin from the stomach of different tuna species. Bars represent standard deviation from triplicate determinations.

Temperature profile of pepsin from 3 tuna species is depicted in Figure 18. The optimal temperature of all pepsin was 50 °C when hemoglobin was used as a substrate. This optimal temperature was slightly higher than those of pepsin or acid proteinase obtained from other warm water fish species such as Monterey sardine (Castillo-Yañez *et al.*, 2004), and sea bream (Zhou *et al.*, 2007). The differences in optimal temperature might be associated with the differences in enzyme conformation as governed by habitat, environment and genetics (Gildberg, 1988). A sharp decrease in activity was observed at temperature above 55 °C, most likely due to thermal denaturation of enzyme. From the result, low activity was observed at 70 °C. At the optimal temperature, the highest specific activity was found in stomach extract from albacore tuna, followed by those from skipjack tuna and tongol tuna, respectively.

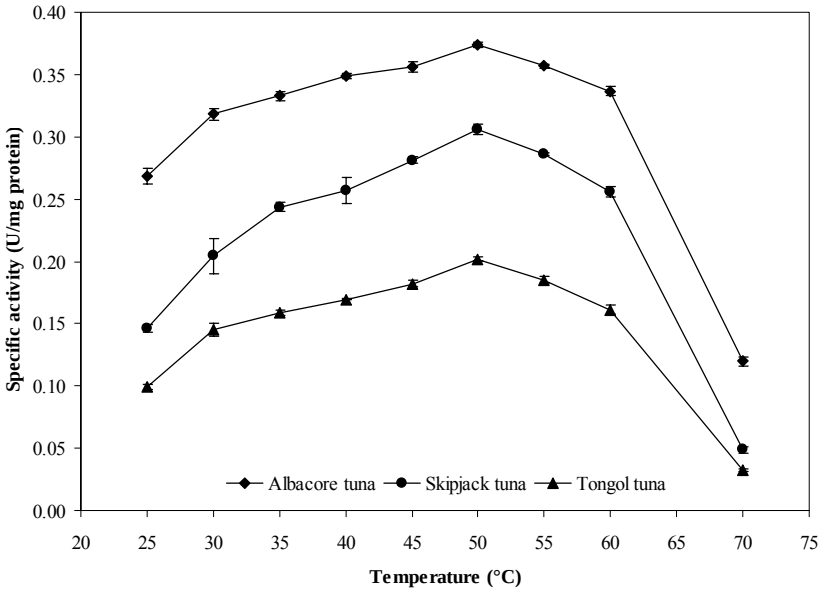

Figure 18. Temperature profiles of pepsin from the stomach of different tuna species. Bars represent standard deviation from triplicate determinations.

5.4.2 Total activity of pepsin from stomach of different tuna species

Total pepsin activities in stomach of 3 tuna species varied ranging from 28.70 to 40.55 units/g. Under the optimal pH and temperature (Figure 17 and 18), albacore tuna stomach contained the highest activity (40.55 units/g) ($P < 0.05$), followed by tongol tuna stomach (37.94 units/g) and skipjack tuna stomach (28.70 units/g). The differences in pepsin content among species might be due to the differences in eating behavior, feed or genetics. Klomklao *et al.* (2004) also found the varying amount of trypsin in spleen among skipjack tuna, tongol tuna, and yellowfin tuna.

5.4.3 Activity staining of tuna pepsin

Tuna pepsins were separated on Native-PAGE, followed by staining for zymogram development (Figure 19). Pepsin from the stomach of albacore tuna and tongol tuna showed a similar pattern of activity band appeared as clear zones on the dark background. Two activity bands with different activity were found as

indicated by differences in clear zone. The result indicated that pepsins in both albacore tuna and tongol tuna most likely consisted of 2 isoforms. For pepsin from skipjack tuna stomach, only one activity band was observed. In general, a major activity band of pepsin from all tuna species appeared at the similar position on zymogram, suggesting the similarity in size and charge distribution among pepsins. Different pepsin isoforms isolated from stomach of different fish species have been reported. Acid proteases I and II from orange roughy (*Hoplostethus atlanticus*) stomach with molecular weight of 33.5 and 34.5 KDa, respectively, were purified (Xu *et al.*, 1996). Two isoforms of pepsins isolated from pectoral rattail (*Coryphaenoides pectoralis*) were classified as pepsin A and B (Klomklao *et al.*, 2007). Two major pepsinogens, PG1 and PG2, and one minor pepsinogen, PG3, were purified from the gastric mucosa of African coelacanth, *Latimeria chalumnae* (Actinistia) (Tanji *et al.*, 2007). Four pepsinogens (PG-I, PG-II, PG-III, and PG-IV) from sea bream (*Sparus latus* Houttuyn) stomach were purified and identified by Zhou *et al.* (2007).

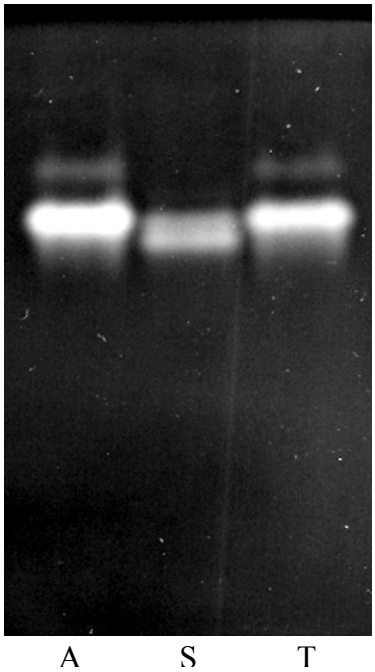

Figure 19. Zymogram of pepsin from the stomach of different tuna species. A: albacore tuna; S: skipjack tuna; T: tongol tuna.

5.4.4 Effect of various protease inhibitors

The effect of various protease inhibitors on the activity of pepsin from different tuna species is shown in Table 13. The activity was completely inhibited by pepstatin A at the concentrations of 1 and 10 μM. Nevertheless, EDTA, soybean trypsin inhibitor and E-64 at the concentrations used had no marked effect on the activity. Pepstatin A inhibits most aspartic proteinase (Umezawa, 1976) with the exception of rennin and retroviral aspartic proteinases (Fusek and Větvička, 1995). Klomklao *et al.* (2007) reported that an activity of both pepsins A and B from pectoral rattail stomach was almost completely inhibited by pepstatin A. Tanji *et al.* (2007) also reported that both pepsins from African coelacanth were nearly completely inhibited by an equimolar amount of pepstatin. E-64, which is specific inhibitor to cysteine proteinase, EDTA, a metalloproteinase inhibitor and soybean trypsin inhibitor, the inhibitor specific to serine proteinase, had no impact on activity. Therefore, the major proteinase in tuna stomach extract was aspartic proteinase, most likely pepsin.

Table 13. Effect of various protease inhibitors on the activity of pepsin from the stomach of different tuna species[†].

Inhibitors	Concentration	Relative activity (%)*		
		Albacore tuna	Skipjack tuna	Tongol tuna
Control		100	100	100
Pepstatin A	1 μM	ND	ND	ND
	10 μM	ND	ND	ND
E-64	0.1 mM	100.3 ± 0.8	97.1 ± 1.9	98.1 ± 1.3
EDTA	0.2 mM	100.3 ± 0.9	97.5 ± 1.0	98.5 ± 0.8
Soybean trypsin inhibitor	0.1 mM	109.1 ± 0.2	105.6 ± 2.7	103.1 ± 0.9

[†] Each enzyme solution was incubated with the same volume of inhibitor at room temperature (26-28 °C) for 20 min and the residual activity was determined using hemoglobin as a substrate for 20 min at pH 2.0 and 50 °C.

* Mean ± S.D. from triplicate determinations.

ND: not detectable.

5.4.5 Effect of some chemicals

The effect of ATP, molybdate, NaCl, $MgCl_2$, $CaCl_2$, SDS, and cysteine on the activity of pepsin from the stomach of different tuna species is shown in Table 14. ATP and molybdate at the concentrations of 0.1 and 0.5 mM had no profound effect on the activity. The result was in agreement with Klomklao *et al.* (2007) who reported that ATP showed no influence on the activity of pepsins from pectoral rattail even though the concentration increased. Cathepsin D is generally activated by ATP, whereas pepsin is not activated (Klomklao *et al.*, 2007; Pillai and Zull, 1985). (Pillai and Zull (1985) found that half-maximal activation of cathepsin D occurs between 200 and 300 μM ATP with all substrate used. From the result, molybdate did not show the inhibition on the activity of tuna stomach extract. Molybdate was reported to inhibit cathepsin D (Pillai and Zull, 1985). Furthermore, all salts tested including NaCl, $CaCl_2$, and $MgCl_2$ also showed no marked influence on the activity of stomach extracts. Tuna pepsin was completely inhibited by SDS at the concentrations of 0.05 and 0.1%, except for pepsin from skipjack tuna which had 29.8% of activity remained in the presence of 0.05% SDS. Cysteine inhibited the activity of stomach extracts in a dose-dependent manner. Pepsins contained 3 disulfide bonds and a phosphate linkage that stabilized their structure together with hydrogen bond, electrostatic, and hydrophobic interactions (Whitaker, 1994). Cysteine acted as a reducing agent, which could destabilize enzyme structure by breaking disulfide bonds.

Table 14. Effect of some chemicals on the activity of pepsin from the stomach of different tuna species†.

Chemicals	Concentration	Relative activity (%)*		
		Albacore tuna	Skipjack tuna	Tongol tuna
Control		100	100	100
ATP	0.1 mM	103.8 ± 1.2	104.3 ± 0.7	104.8 ± 2.0
	0.5 mM	104.4 ± 0.5	106.5 ± 1.3	106.8 ± 1.3
Molybdate	0.1 mM	105.0 ± 0.2	102.7 ± 0.9	105.7 ± 0.6
	0.5 mM	105.0 ± 0.8	105.0 ± 0.7	105.4 ± 2.2
NaCl	1 mM	99.7 ± 0.6	105.9 ± 1.1	101.9 ± 0.8
	5 mM	103.7 ± 0.3	109.1 ± 2.7	106.5 ± 1.4
$MgCl_2$	1 mM	100.4 ± 0.8	103.9 ± 1.0	103.2 ± 1.6
	5 mM	102.2 ± 0.4	103.6 ± 1.8	103.2 ± 0.3
$CaCl_2$	1 mM	101.2 ± 0.4	102.6 ± 0.4	103.2 ± 1.5
	5 mM	105.6 ± 1.3	105.7 ± 1.0	106.1 ± 0.6
SDS	0.05% (w/v)	ND	29.8 ± 1.3	ND
	0.10% (w/v)	ND	ND	ND
Cysteine	5 mM	97.1 ± 0.6	97.2 ± 0.5	98.6 ± 0.4
	25 mM	94.4 ± 0.5	81.9 ± 1.4	76.4 ± 1.6
	50 mM	36.8 ± 5.4	33.5 ± 1.7	35.5 ± 3.4

† Each enzyme solution was incubated with the same volume of chemical at room temperature (26-28 °C) for 30 min and the residual activity was determined using hemoglobin as a substrate for 20 min at pH 2.0 and 50 °C.

* Mean ± S.D. from triplicate determinations.

ND: not detectable.

5.4.6 Use of tuna pepsin for collagen extraction

The yields of collagen extracted from threadfin bream skin using pepsin from stomach of different tuna species for different times (6 and 12 h) in comparison with acid solubilization process (without pepsin) are shown in Table 15. The yields of collagen extracted with the aid of porcine pepsin at the same levels were also compared (Table 15). The highest yield (~74.5-76%) was obtained when the skin was extracted using pepsin from albacore tuna or porcine pepsin at a level of 10 units/g of defatted skin for 12 h. At the same extraction time, the use of tuna pepsins and porcine pepsin resulted in the higher yield than the control (without pepsin). In general, longer reaction time afforded a higher yield, regardless of pepsin addition. The yield of collagen extracted by pepsin treatments for 12 h was 1.84-2.38

and 0.83-1.17 fold greater than that obtained from acid solubilization process with the extraction time of 12 and 48 h, respectively. It was suggested that hydrolysis at the telopeptide region was more pronounced with sufficient reaction time. As a consequence, collagen could be solubilized into the extracting solution in the presence of both tuna and porcine pepsins to a greater extent, compared with the collagen extracted without pepsins. This result was in agreement with Nalinanon *et al.* (2007) who found that the addition of bigeye snapper pepsin and porcine pepsin at a level of 20 kUnits/g defatted skin resulted in the increased content of collagen extracted from bigeye snapper skin. Mammalian pepsin has been used for collagen extraction from fish skin, including ocellate puffer fish (Nagai *et al.*, 2002), black drum, sheephead seabream (Ogawa *et al.*, 2003), and channel catfish (Liu *et al.*, 2007). From the result, albacore tuna pepsin exhibited higher efficiency in collagen extraction, compared with skipjack tuna pepsin ($P < 0.05$), and showed similar effectiveness in increasing the yield to porcine pepsin and tongol pepsin. The differences in collagen yield were obtained when pepsins from different sources were used, suggesting the differences in the cleavage of telopeptide of collagen from threadfin bream skin. Due to the similar extraction efficiency, pepsin from albacore tuna could be used as a substitute for porcine pepsin.

Table 15. Total hydroxyproline (Hyp) and yield of collagen extracted from the skin of threadfin bream with the aid of pepsin from the stomach of different tuna species and porcine pepsin for different times.

Treatments[†]	Extraction time (h)	Total extracted Hyp* (mg/g defatted skin)	Yield[‡] (%)
Control (without pepsin)	6	2.19 ± 0.41^{a}	$12.32 \pm 2.29^{a\#}$
	12	4.00 ± 0.27^{b}	22.45 ± 1.50^{b}
	48	6.22 ± 0.15^{c}	34.90 ± 0.82^{c}
Albacore pepsin	6	10.55 ± 0.35^{f}	59.22 ± 1.95^{f}
	12	13.26 ± 0.24^{hi}	74.48 ± 1.35^{hi}
Skipjack pepsin	6	7.78 ± 0.22^{d}	43.68 ± 1.25^{d}
	12	11.36 ± 0.08^{g}	63.81 ± 0.44^{g}
Tongol pepsin	6	9.69 ± 0.21^{e}	54.41 ± 1.15^{e}
	12	12.81 ± 0.45^{h}	71.95 ± 2.53^{h}
Porcine pepsin	6	9.36 ± 0.53^{e}	52.56 ± 2.95^{e}
	12	13.52 ± 0.19^{i}	75.92 ± 1.05^{i}

Means ± SD from triplicate determinations.

[†] Pepsin at a level of 10 units/g of defatted skin was used.

[‡] Yield was calculated, based on hydroxyproline content in the collagen in comparison with that of defatted skin.

[#] Different superscripts in the same column indicate the significant differences ($P < 0.05$).

* Hydroxyproline content in the skin of threadfin bream was 17.81 mg/g defatted skin.

Electrophoretic study of collagens extracted under different conditions revealed that β, α1 and α2 were the major components and collagen obtained was classified as type I (Figure 20). The result was accordance with those found in skin collagen of black drum and sheephead seabream (Ogawa *et al.*, 2003), Pacific whiting (Kim and Park, 2004), young and adult Nile perch (Muyonga *et al.*, 2004), channel catfish (Liu *et al.*, 2007), and bigeye snapper (Nalinanon *et al.*, 2007). Generally, type I collagen, a major collagen in fish skin, consists of 2 identical α1 chain and one α2 chain (Rochdi *et al.*, 2000; Yata *et al.*, 2001). No differences in protein pattern were noticeable among collagens obtained from different extraction processes, excepted that treated with pepsin from skipjack tuna. Most protein bands were degraded with

the concomitant formation of low MW peptides or proteins when pepsin from skipjack tuna was used. It was suggested that pepsin from skipjack tuna not only hydrolyzed peptides at telopeptide region, but also within tropocollagen as indicated by the decreased band intensity of β and α chains. Generally, the higher molecular mass components including γ-chain were found at the higher extent in acid-solubilized collagen, compared with those observed in pepsin-solubilized collagens. When reaction time increased, the intensity of β-chain of pepsin-solubilized collagen was noticeably increased, possibly due to the cleavage of higher MW components. Similar changes were also found in calf skin tropocollagen (Drake *et al.*, 1966), deep-sea redfish (Wang *et al.*, 2007) and bigeye snapper skin collagen (Nalinanon *et al.*, 2007) when pepsin was used for collagen extraction. Drake *et al.* (1966) reported that native calf skin tropocollagen consisted of α:β:γ at a ratio of 32:65:3, whereas the components of pepsin treated tropocollagen were changed to 72:23:3. Wang *et al.* (2007) also found that the band intensity ratios of cross-linked chain (β and γ chains) to total monomer chain (α1 + α2) were lowered in pepsin-solubilized collagen extracted from deep-sea redfish when compared with acid-solubilized collagen. This result indicated that pepsin used in this study could cleave intermolecular bonds of collagen specifically. Zimmermann *et al.* (1970) demonstrated that 2 types of intermolecular bonds, side-to-side and end-to-end bond, in calf collagen can easily be cleaved by pepsin, whereas head-to-tail bond is relatively pepsin resistant.

It was noted that similar MW of α chains were observed between acid solubilized collagen and tuna-pepsin solubilzed collagen. However, Nalinanon *et al.* (2007) reported that MW of α chains of collagen from bigeye snapper skin extracted with pepsin from the same fish species decreased slightly, when compared with acid-solubilized collagen. Therefore, it was likely that the use of pepsin from the same fish species to extract the fish skin might cause more intensive cleavage at telopeptide region. Therefore, pepsins from both albacore and tongol could be used to extract collagen from threadfin bream skin effectively without the alteration of MW of components in resulting collagen.

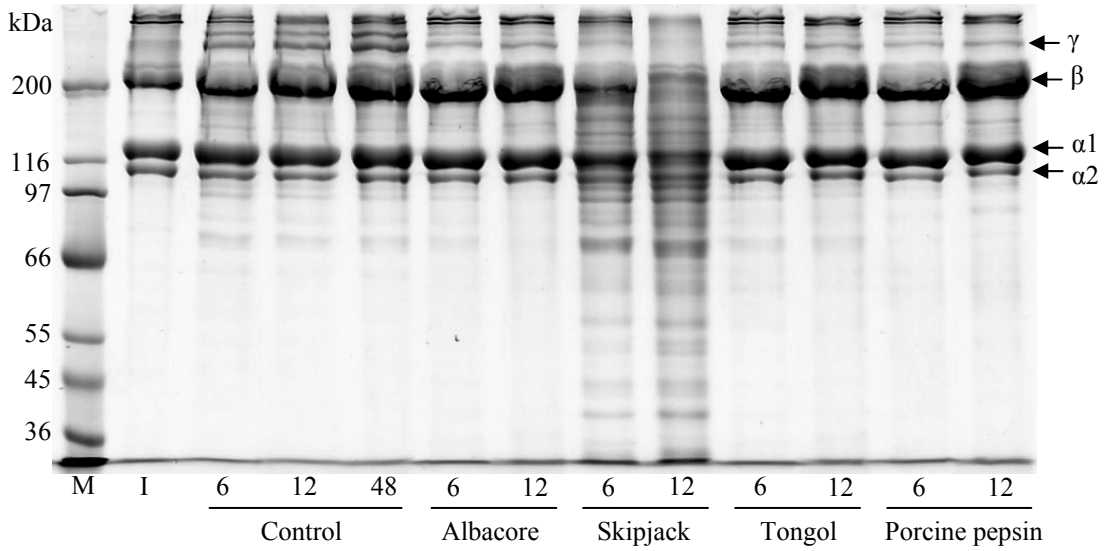

Figure 20. SDS–PAGE patterns of collagens extracted from threadfin bream skin with the aid of pepsin from the stomach of different tuna species and porcine pepsin (10 units/g defatted skin) for different extracting times. Numbers denote extraction time (h). M and I denote MW protein markers and collagen type I, respectively.

5.5 Conclusion

Tuna pepsins showed optimal activity at pH 2 and 50 °C. The activity was strongly inhibited by pepstatin A, SDS, and cysteine. Among all tuna species, albacore tuna contains the highest pepsin activity ($P < 0.05$). Collagen extraction from threadfin bream skin could be achieved by soaking the prepared skin in 0.5 M acetic acid containing albacore pepsin at a level of 10 units/g defatted skin for 12 h. Albacore pepsin exhibited the comparable extraction efficiency to porcine pepsin and no detrimental effect on the integrity of resulting collagen was observed. Therefore, albacore pepsin could be used as the potential aid for collagen extraction from threadfin bream skin.

CHAPTER 6

COLLAGEN FROM THE SKIN OF UNICORN LEATHERJACKET (*ALUTERUS MONOCEROS*) SOLUBILIZED BY ALBACORE TUNA PEPSIN

6.1 Abstract

Collagen from the skin of unicorn leatherjacket (*Aluterus monoceros*) was extracted without and with the aid of pepsin from albacore tuna (*Thunnus alalunga*) at a level of 20 units/g residual skin. The yield of pepsin solubilized collagen (PSC) (28.33% dry wt.) was approximately 8-fold higher than that of acid solubilized collagen (ASC). Both ASC and PSC consisted of α, β and γ components. The collagens were characterized to be type I with no disulfide bond. A slight decrease in molecular weight of α-chains of PSC was observed, in comparison with that of ASC. ASC and PSC contained high content of imino acids (~187 residues/1000 residues). Native collagens were resistant to chymotrypsin digestion, however, collagens preheated at 50 °C were susceptible to chymotrypsin. The degradation induced by chymotrypsin was more pronounced in both ASC and PSC, compared with calf skin collagen. The maximum transition temperatures (T_m) of ASC and PSC were 30.18 °C and 30.03 °C, respectively. Fourier transform infrared (FTIR) spectroscopy revealed that the triple helical structure was predominant in both collagens but there was a slight difference in secondary structure between both collagens. Based on ζ-potential, p*I* of ASC and PSC was observed at 6.29 and 6.37, respectively. Therefore, the increase in extraction efficiency of collagen from unicorn leatherjacket was achieved with the aid of albacore tuna pepsin without the marked effect on the structure of resulting collagen.

6.2 Introduction

Collagen is a major structural protein in the connective tissue of animal skin and bone. Generally, collagen has a wide range of applications in cosmetic, biomedical and pharmaceutical industries (Jongjareonrak *et al.*, 2005; Nalinanon *et al.*, 2007). Fish skin can serve as an alternative source for collagen since mammalian collagens are associated with several problems such as the outbreak of mad cow disease and the constraint for some religions, mainly Islam and Judaism (Nalinanon *et al.*, 2008b). Therefore, skin collagens from several fish species such as bigeye snapper (Kittiphattanabawon *et al.*, 2005; Nalinanon *et al.*, 2007), black drum (Ogawa *et al.*, 2003), brownstripe red snapper (Jongjareonrak *et al.*, 2005), carp (Duan *et al.*, 2009), channel catfish (Liu *et al.*, 2007), Nile perch (Muyonga *et al.*, 2004), ocellate puffer fish (Nagai *et al.*, 2002), sheephead seabream (Ogawa *et al.*, 2003), threadfin bream (Nalinanon *et al.*, 2008b) and yellowfin tuna (Woo *et al.*, 2008) have been extracted and characterized.

Generally, the typical process, in which an acid solubilization is implemented, renders a low yield of collagen. To tackle the problem, pepsin has been applied since it is able to cleave peptides specifically in telopeptide region of collagen, leading to an increased extraction efficiency (Nalinanon *et al.*, 2007; Nalinanon *et al.*, 2008b). Pepsin has been successfully used for collagen extraction from marine sources. The yield of PSC from the skin of ocellate puffer fish (44.7% dry wt.) was much higher than that of ASC (10.7% dry wt.) (Nagai *et al.*, 2002). The higher yield of PSC from the skin of black drum and sheephead seabream (Ogawa *et al.*, 2003) and channel catfish (Liu *et al.*, 2007), in comparison with ASC, was also reported. However, all pepsins used were porcine pepsins, which are associated with a limitation for some religions. Recently, fish pepsins including tuna pepsins and bigeye snapper pepsin have been effectively used to increase the yield of collagen (Nalinanon *et al.*, 2007; Nalinanon *et al.*, 2008b).

Due to the continuous decrease in economically important fish, other fish species have been paid more attention, especially for export. Unicorn leatherjacket is a species prevalent in both Andaman Sea and the Gulf of Thailand and has been used for fillet preparation, owing to its firm texture and white color. As a

consequence, skin is produced as by-product, which can be used as a promising source of collagen. However, the use of pepsin might be required to increase the extraction efficiency of collagen from the skin of this species, which is thick and tough in nature. To lower the cost of commercial pepsin, tuna pepsin which could be recovered from stomach can be used as the cheap source of pepsin. Therefore, the objectives of this study were to extract collagen from the skin of unicorn leatherjacket with the aid of albacore tuna pepsin and to characterize the resultant collagens in comparison with that obtained from acid solubilization process.

6.3 Materials and Methods

6.3.1 Chemicals

Bovine hemoglobin, *β*-mercaptoethanol (βME), *L*-tyrosine and wide range protein markers were purchased from Sigma Chemical Co. (St. Louis, MO, USA.). α-Chymotrypsin from bovine pancreas (EC 3.4.21.1, 1,000 USP chymotrypsin units/mg) was procured from Wako Pure Chemical Industries, Ltd. (Osaka, Japan). Trichloroacetic acid, disodium hydrogen phosphate, sodium citrate, Folin-Ciocalteu's phenol reagent, acetic acid, and *p*-dimethylamino-benzaldehyde were obtained from Merck (Darmstadt, Germany). Coomassie Blue R-250 and *N,N,N′,N′*-tetramethyl ethylene diamine (TEMED) were procured from Bio-Rad Laboratories (Hercules, CA, USA). Sodium dodecyl sulfate (SDS) and bovine serum albumin were obtained from Fluka (Buchs, Switzerland). Type I collagen from calf skin was purchased from Elastin Products Co., Inc. (Owensville, MO, USA).

6.3.2 Preparation of fish stomach and skin

Internal organ of albacore tuna (*Thunnus alalunga*) was obtained from Tropical Canning (Thailand) Public Co., Ltd., Songkhla, Thailand. The sample was packed in polyethylene bag, kept in ice with a solid/ice ratio of 1:2 (w/v) and transported to the Department of Food Technology, Prince of Songkla University, Hat Yai within 1 h. Upon the arrival, a pooled internal organ was excised and only

stomach was collected and placed in polyethylene bag. The stomach sample was stored at -20 °C until use.

Unicorn leatherjacket (*Aluterus monoceros*) was obtained after being off-loaded for 24 h from the dock in Songkhla, Thailand. The skin was removed and kept in ice and transported to the Department of Food Technology within 1 h. Upon the arrival, the skin was washed with tap water, drained and cut into small pieces (0.5 × 0.5 cm) using the scissor. The prepared skins were placed in polyethylene bag and stored at -20 °C until use.

6.3.3 Preparation of pepsin from albacore tuna

Frozen stomach was thawed using running water (26-28 °C) until the core temperature reached -2 to 0 °C. The sample was cut into pieces with a dimension of 0.5-0.5 cm^2. Sample was rapidly frozen in liquid nitrogen and then finely ground using a National Model MX-T2GN blender (Taipei, Taiwan) to a powder form. Stomach powder was suspended in 50 mM sodium phosphate buffer, pH 7.2 at a ratio of 1:9 (w/v) according to the method of Nalinanon *et al.* (2007). The mixture was stirred continuously at 4 °C for 30 min. The suspension was centrifuged at 8,000 × *g* for 30 min at 4 °C using a Sorvall Model RC-B Plus refrigerated centrifuge (Newtown, CT, USA) to remove the tissue debris. The supernatant was collected and then adjusted to pH 2 with 1 M HCl. The mixture was allowed to stand at 4 °C for 30 min. The treated stomach extract was then centrifuged at 10,000 × *g* for 30 min at 4 °C using a refrigerated centrifuge. The acidified supernatants were collected and referred to as “pepsin extract”.

6.3.4 Enzyme assay

Proteolytic activity of pepsin extract from albacore tuna was determined using hemoglobin as a substrate according to the method of Nalinanon *et al.* (2008b). To initiate the reaction, 200 µl of pepsin extract was added into the assay mixture containing 200 µl of 2% hemoglobin, 200 µl of distilled water and 625 µl of

McIlvaine's buffer (0.2 M Na-phosphate and 0.1 M Na-citrate), pH 2.0. Appropriate dilution was made to ensure that the amount of enzyme was not excessive for available substrate in the assay system. The reaction was conducted at 50 °C for 20 min. To terminate enzymatic reaction, 200 µl of 50% (w/v) trichloroacetic acid (TCA) were added. Unhydrolyzed protein substrate was allowed to precipitate for 15 min at 4 °C, followed by centrifuging at 4,725 × *g* for 10 min using a MIKRO 20 centrifuge (Hettich Zentrifugen, Tuttlingen, Germany). The oligopeptide content in the supernatant was determined by the Lowry method (Lowry *et al.*, 1951) using tyrosine as a standard. One unit of activity was defined as that releasing 1 µmole of tyrosine per min (µmol Tyr/min). A blank was run in the same manner, except that pepsin extract was added into the reaction mixture after the addition of 50% TCA (w/v).

6.3.5 Preparation of skin collagen

The collagens from the skin of unicorn leatherjacket were isolated following the method of Nalinanon *et al.* (2007) and Jongjareonrak *et al.* (2005) with a slight modification. All procedures were performed at 4 °C. To remove non-collagenous proteins, the skin was mixed with 0.1 N NaOH using a sample/alkaline solution ratio of 1:10 (w/v). The mixture was stirred continuously for 6 h. The alkaline solution was changed every 2 h. Then, the alkaline treated skin was washed with cold water until neutral or faintly basic pHs of wash water were obtained. The treated skin was then defatted with 10% (v/v) 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 3 times.

To extract the collagen, the prepared skin was soaked in 0.5 M acetic acid with a sample/solution ratio of 1:15 (w/v) for 48 h with gentle stirring. Then, the mixture was filtered with 2 layers of cheesecloth to remove undissolved tissues. The filtrate was precipitated by the addition of NaCl to a final concentration of 2.6 M in 0.05 M Tris–HCl (pH 7.5). The resultant precipitate was collected by centrifuging at 20,000 ×*g* for 60 min. The pellet was dissolved in 10 volumes of 0.5 M acetic acid. The solution obtained was dialyzed against 10 volumes of 0.1 M acetic acid in a

dialysis bag with a molecular weight cut-off of 14 kDa for 12 h with the change of dialysis solution every 4 h. Subsequently, the solution was dialyzed with 30 volumes of distilled water with the changes of water until neutral pH was obtained. The dialysate was freeze-dried and referred to as 'acid solubilized collagen, ASC'. Undissolved residue obtained after acid extraction was suspended in 0.5 M acetic acid with a solid/solvent ratio of 1:15 (w/v) in the presence of albacore tuna pepsin with the activity of 20 units/g residual skin. The mixture was gently stirred for 48 h, followed by filtration using two layers of cheesecloth. Then, the mixture was immediately precipitated by the addition of NaCl to a final concentration of 2.6 M in 0.05 M Tris–HCl (pH 7.5) and allowed to stand for 1 h for pepsin inactivation. The resultant precipitate was collected by centrifugation at 20,000 ×*g* for 1 h and re-dissolved in 10 volumes of 0.5 M acetic acid. The solution was dialyzed and freeze-dried in the same manner as for ASC preparation. Dry matter was referred to as 'pepsin solubilized collagen, PSC'. ASC and PSC were subjected to analyses. The yield of collagen was calculated based on lyophilized weight of resulting collagen in comparison with that of dried defatted skin.

6.3.6 Characterization of collagen

6.3.6.1 Amino acid analysis

ASC and PSC 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).

6.3.6.2 SDS–polyacrylamide gel electrophoresis (SDS–PAGE)

SDS–PAGE was performed by the method of Laemmli (1970). The collagen samples were dissolved in 0.02 M sodium phosphate containing 1% SDS and 3.5 M urea (pH 7.2). The mixtures were centrifuged at 8500 ×*g* for 5 min at room temperature to remove undissolved debris. Solubilized samples were mixed at 1:1 (v/v) ratio with the sample buffer (0.5 M Tris–HCl, pH 6.8, containing 4% SDS, 20%

glycerol) in the presence or absence of 10% β-ME. Samples were loaded onto a polyacrylamide gel made of 7.5% running gel and 4% stacking gel and subjected to electrophoresis at a constant current of 15 mA/gel, using a Mini Protein II unit (Bio-Rad Laboratories Inc., Richmond, CA, USA). After electrophoresis, the gel was stained with 0.05% (w/v) Coomassie Blue R-250 in 15% (v/v) methanol and 5% (v/v) acetic acid and destained with 30% (v/v) methanol and 10% (v/v) acetic acid. Wide range protein markers (Sigma Chemical Co., St. Louis, MO, USA) were used to estimate the molecular weights of proteins. Type I collagen from calf skin was used as a standard.

6.3.6.3 Differential scanning calorimetry (DSC)

Prior to analysis, collagen samples were prepared following the methods of Rochdi *et al.* (2000) and Komsa-Penkova *et al.* (2000) with a slight modification. The samples were rehydrated by adding 0.05 M acetic acid to dried samples at a solid/solution ratio of 1:40 (w/v). The mixture was allowed to stand for 2 days at 4 °C.

Differential scanning calorimetry (DSC) was performed using a differential scanning colorimeter (Model DSC 7, Norwalk, Connecticut, USA). Temperature calibration was done using the indium thermogram. The collagen solutions (5–10 mg) were accurately weighed into aluminium pans and sealed. The samples were scanned at 1 °C/min over the range of 20–50 °C using ice water as the cooling medium. An empty pan was used as the reference. The maximum transition temperature (T_m) was estimated from the DSC thermogram.

6.3.6.4 Fourier transform infrared (FTIR) spectroscopy

Spectra of ASC and PSC from the skin of unicorn leatherjacket were obtained by using a Bruker Model EQUINOX 55 FTIR spectrometer (Bruker, Ettlingen, Germany) equipped with a deuterated l-alanine tri-glycine sulfate (DLATGS) detector. The Horizontal Attenuated Total Reflectance Accessory (HATR) was mounted into the sample compartment. The internal reflection crystal (Pike Technologies, Madison, WI, USA), which was made of zinc selenide, had a 45° angle of incidence to the IR beam. Spectra were acquired at a resolution of 4 cm^{-1} and

the measurement range was 4000–800 cm^{-1} (mid-IR region) at room temperature. Automatic signals were collected in 16 scans at a resolution of 4 cm^{-1} and were ratioed against a background spectrum recorded from the clean, empty cell at 25 °C. Analysis of spectral data was carried out using the OPUS 3.0 data collection software program (Bruker, Ettlingen, Germany).

6.3.6.5 Chymotryptic hydrolysis of skin collagen

Samples (5 mg) were suspended in 0.6 mL of 50 mM Tris-HCl, pH 8.0, containing 10 mM $CaCl_2$. The collagen mixture was incubated at 25 °C or 50 °C for 30 min. After incubation, 50 μL of the same buffer containing 5 μg of chymotrypsin was added to collagen solutions and was then incubated at 25 °C for 15 min. The reaction was terminated by adding 650 μL of 5% (w/v) SDS (85 °C) to the reaction mixture and boiling for 10 min. Peptides generated by the chymotrypsin digestion were subjected to SDS–PAGE using 7.5% running gel and 4% stacking gel as previously described. Peptide mapping of calf skin collagen acid-soluble type I was conducted in the same manner and the peptide patterns were compared.

6.3.6.6 Measurement of zeta (ζ) potential

Collagen samples were dissolved in 0.5 M acetic acid at a concentration of 0.5 mg/mL. The mixture was stirred at 4 °C for 12 h. The ζ-potential of each sample (20 mL) was measured using a Zeta potential analyzer (ZetaPALS, Brookhaven Instruments Co., Holtsville, New York, USA). ζ-potential of samples adjusted to different pHs with 1.0 M Nitric acid or 1.0 M KOH using an Autotitrator (BI-ZTU, Brookhaven Instruments Co., Holtsville, New York, USA) was determined. The isoelectric point (p*I*) was estimated from pH rendering zero ζ-potential.

6.3.7 Statistical analysis

All data were subjected to Analysis of Variance (ANOVA) and the differences between means were evaluated by Duncan's multiple range test (Steel and Torrie, 1980). SPSS statistic program (SPSS 11.0 for Windows, SPSS Inc., Chicago, IL, USA) was used for data analysis.

6.4 Results and Discussion

6.4.1 Yield of collagen from the skin of unicorn leatherjacket

ASC was extracted with acetic acid and PSC was extracted from the residue of ASC extraction. The yields of ASC and PSC were 3.39 and 28.33% (dry wt.), respectively. The result indicated that the yield of collagen was noticeably increased ($P < 0.05$), approximately by 8-fold when albacore tuna pepsin was used to extract the residual collagen after acid extraction. The skin collagen of unicorn leatherjacket was hardly solubilized with 0.5 M acetic acid but it was easily solubilized by the limited pepsin hydrolysis. The swollen skins after acid extraction were easily cleaved with pepsin at telopeptide regions (Nalinanon *et al.*, 2007). Therefore, albacore tuna pepsin was effective in solubilizing collagen from the skin of unicorn leatherjacket. This result was in accordance with those found in collagen extractions from the skin of bigeye snapper (Nalinanon *et al.*, 2007) and threadfin bream (Nalinanon *et al.*, 2008b) when fish pepsin was used as the extraction aid. Nalinanon *et al.* (2007) found that the addition of bigeye snapper pepsin and porcine pepsin at a level of 20 units/g defatted skin resulted in the increased yield of collagen extracted from bigeye snapper skin. When tuna pepsin (10 units/g defatted skin) was used for collagen extraction from the skin of threadfin bream for 12 h, the yield of collagen increased by 1.84-2.32-fold and albacore pepsin showed the comparable extraction efficacy to porcine pepsin (Nalinanon *et al.*, 2008b).

6.4.2 Protein patterns of collagen from the skin of unicorn leatherjacket

Protein patterns of ASC and PSC, under reducing and non-reducing conditions, are shown in Figure 21. Generally, no differences in protein patterns of both collagens under both conditions were observed. This result indicated that both ASC and PSC contained no disulfide bonds. Both collagens comprised β, α1 and α2 chains as the major constituents. The results was in accordance with those found in skin collagen of bigeye snapper (Kittiphattanabawon *et al.*, 2005; Nalinanon *et al.*, 2007), brownstripe red snapper (Jongjareonrak *et al.*, 2005), carp (Duan *et al.*, 2009),

channel catfish (Liu *et al.*, 2007), threadfin bream (Nalinanon *et al.*, 2008b), black drum and sheepshead seabream (Ogawa *et al.*, 2003). In general, ASC and PSC showed similar protein pattern. Based on electrophoretic patterns and subunit composition, ASC and PSC from the skin of unicorn leatherjacket were most likely classified to be type I collagen, which consists of 2 identical α1 chains and 1 α2 chain (Nalinanon *et al.*, 2008b; Rochdi *et al.*, 2000). However, slight difference in relative mobility between both different collagens was observed. The molecular weight (MW) of α1 and α2 chains of ASC was estimated to be 121 and 112 kDa, respectively. For PSC, MW of α1 and α2 chains was 118 and 109 kDa, respectively. It was noted that a slight decrease in MW of α-chains of PSC was observed, in comparison with that of ASC. The result suggested that PSC might undergo the partial cleavage at telopeptide regions by pepsin treatment. The similar result was previously found in the collagen extraction from the skin of bigeye snapper using pepsin from bigeye snapper stomach (Nalinanon *et al.*, 2007). Collagen type I from calf skin consisted of α1 and α2 chains with MW of 123 and 114 kDa, respectively. MW of calf skin collagen was slightly higher than that of collagens from the skin of unicorn leatherjacket. Drake *et al.* (1966) reported that some of the telopeptides of calf skin tropocollagen are susceptible to pepsin hydrolysis and intramolecular cross-links are broken on pepsin digestion.

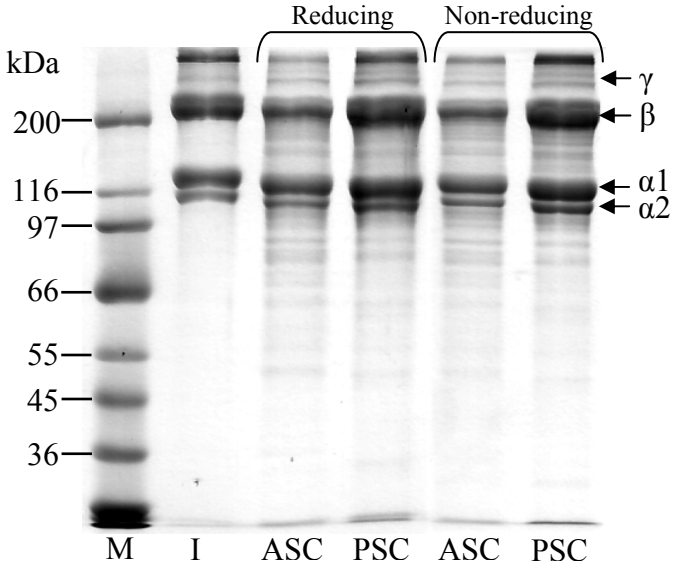

Figure 21. SDS-PAGE patterns of acid solubilzed collagen (ASC) and pepsin solubilized collagen (PSC) from the skin of unicorn leatherjacket under reducing and non-reducing conditions. M and I denote wide range MW protein markers and collagen type I, respectively.

6.4.3 Amino acid composition

The amino acid composition of ASC and PSC is expressed as residues per 1000 total amino acid residues as shown in Table 16. Generally, ASC and PSC extracted from unicorn leatherjacket had similar amino acid composition. Glycine was the most abundant amino acid followed by alanine, proline, glutamic acid and hydroxyproline, respectively. Both collagens contained no tryptophan. Cysteine, tyrosine and histidine were found with a low content. Both ASC and PSC consisted of proline and hydroxyproline, which are unique amino acids found in collagen (Jongjareonrak *et al.*, 2005). The imino acid contents (proline and hydroxyproline) of ASC and PSC from unicorn leatherjacket skin were 187 and 186 residues/1000 residues, respectively, which was similar to those of grass carp skin collagen (186 residues/1000 residues) (Zhang *et al.*, 2007). The imino acid contents of both ASC and PSC were relatively higher than those of skin collagens from cold-water fish, such as cod (154 residues/1000 residues) (Giraud-Guille *et al.*, 2000) and ocellate

puffer fish (170 residues/1000 residues) (Nagai *et al.*, 2002). However, imino acid contents of both ASC and PSC from unicorn leatherjacket skin were lower than those reported for collagen from the skin of carp (Duan *et al.*, 2009), black drum and sheephead (Ogawa *et al.*, 2003), brownstripe red snapper (Jongjareonrak *et al.*, 2005) and bigeye snapper (Kittiphattanabawon *et al.*, 2005), which contained imino acids ranging from 190 to 221 residues/1000 residues. Proline and hydroxyproline contents vary with species and their living habitat (Foegeding *et al.*, 1996; Jongjareonrak *et al.*, 2005; Kittiphattanabawon *et al.*, 2005). Imino acids contribute to the stability of helix structure of collagen (Ikoma *et al.*, 2003; Jongjareonrak *et al.*, 2005). Pyridinoline rings of proline and hydroxyproline impose restrictions on the conformation of the polypeptide chain and help to strengthen the triple helix (Bae *et al.*, 2008). Hydroxylysine of 4-5 residues/1000 residues was found in both collagens, suggesting the partial cross-linking of collagen via covalent bond (Mechanic *et al.*, 1987). Therefore, collagen from unicorn leather jacket might have the different molecular properties from that of other species due to the difference in imino acid content and cross-linking.

Table 16. Amino acid composition of ASC and PSC from the skin of unicorn leatherjacket (residues/1000 residues)

Amino acid	ASC	PSC
Alanine	140	139
Arginine	53	54
Aspartic acid/asparagine	48	45
Cysteine	1	1
Glutamic acid/glutamine	75	74
Glycine	325	325
Histidine	6	7
Isoleucine	8	9
Leucine	17	17
Lysine	28	28
Hydroxylysine	4	5
Methionine	10	12
Phenylalanine	12	13
Hydroxyproline	71	71
Proline	116	115
Serine	33	34
Threonine	27	28
Tyrosine	4	3
Tryptophan	0	0
Valine	21	22
Total	**1000**	**1000**
Imino acids*	187	186

* Imino acids include proline and hydroxyproline

6.4.4 Thermal stability

DSC thermograms of ASC and PSC rehydrated in 0.05 M acetic acid are shown in Figure 22. Endothermic peaks with the peak maximum temperature (T_m) of 30.18 and 30.03 °C, were observed for ASC and PSC, respectively. T_m of ASC from the skin of unicorn leatherjacket was similar to that of PSC, suggesting no differences in the denaturation temperature between the both collagens. Therefore, pepsin digestion did not affect the denaturation temperature of PSC, though a slight decrease in MW was obtained. It was suggested that pepsin specifically hydrolyzed telopeptide region without any cleavage of α-chains. However, the decrease in denaturation temperature about 1°C was reported for PSC from bigeye snapper skin, compared to ASC (Nalinanon *et al.*, 2007). T_m of collagens from the skin of unicorn

leatherjacket was similar to that of brownstripe red snapper (30.5 °C) (Jongjareonrak *et al.*, 2005). However, it was lower than those of skin collagens from bigeye snapper (32.5 °C) (Nalinanon *et al.*, 2007), channel catfish (32.5 °C) (Liu *et al.*, 2007), Nile perch (36 °C) (Muyonga *et al.*, 2004), eagle ray (34.1 °C) (Bae *et al.*, 2008), black drum (34.2 °C) (Ogawa *et al.*, 2003), sheephead seabream (34 °C) (Ogawa *et al.*, 2003) and porcine (37 °C) (Liu *et al.*, 2007; Nagai *et al.*, 2002). Lower denaturation temperatures of skin collagen from several cold and temperate water fishes have been reported, including carp (28 °C), cod (15 °C) (Duan *et al.*, 2009), grass carp (28.4 °C) (Zhang *et al.*, 2007), deep-sea redfish (16.1 °C) (Wang *et al.*, 2007), ocellate puffer fish (28 °C) (Nagai *et al.*, 2002), tiger puffer fish (28.4 °C) (Bae *et al.*, 2008), Japanese seabass (26.5 °C), chub mackerel (25.6 °C) and bullhead shark (25 °C) (Nagai and Suzuki, 2000). The differences in denaturation temperature of collagen from different sources might be determined by different contents of imino acids (proline and hydroxyproline). Imino acid content shows a direct positive correlation with the thermal stability of protein via the formation of hydrogen bonds, through the hydroxyl group of hydroxyproline (Jongjareonrak *et al.*, 2005; Sikorski *et al.*, 1984; Zhang *et al.*, 2007). Proline stabilizes the helix structure by preventing rotation of the N-C bond (Foegeding *et al.*, 1996). Furthermore, imino acid content of fish collagen, and their thermal stability, correlates with the water temperature of their normal habitat (Foegeding *et al.*, 1996).

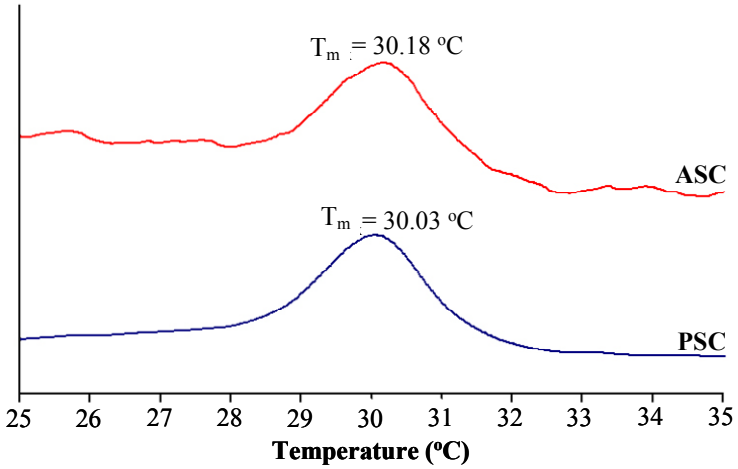

Figure 22. DSC thermograms of acid solubilized collagen (ASC) and pepsin solubilized collagen (PSC) from the skin of unicorn leatherjacket. Collagen samples were rehydrated in 0.05 M acetic acid prior to analysis.

6.4.5 FTIR spectra of collagen from the skin of unicorn leatherjacket

FTIR spectra of ASC and PSC from the skin of unicorn leatherjacket are shown in Figure 23. The skin collagens from unicorn leatherjacket exhibited FTIR spectra similar to that found in other skin collagens (Duan *et al.*, 2009; Liu *et al.*, 2007; Muyonga *et al.*, 2004; Wang *et al.*, 2007; Woo *et al.*, 2008), in which the absorption bands in the spectra were situated in the amide band region including amide A, amide I, amide II and amide III. Amide A band (3400-3440 cm^{-1}) is related to N-H stretching vibrations (Woo *et al.*, 2008). When the N-H group of peptide is involved in a hydrogen bond, the position (3300 cm^{-1}) is shifted to lower frequency (Duan *et al.*, 2009; Wang *et al.*, 2007). The amide A of ASC was found at 3305 cm^{-1}, while PSC was at 3307 cm^{-1}. This indicated that more N-H group of ASC was involved in hydrogen bond (Wang *et al.*, 2007). The absorption associated with the Amide I band (1600-1660 cm^{-1}) due to stretching vibrations of the carbonyl group (C=O bond) along the polypeptide backbone has been used to investigate the secondary structure of a protein (Wang *et al.*, 2007; Woo *et al.*, 2008). Amide II (~1550 cm^{-1}) is associated with N-H bending coupled with C-N stretching (Muyonga *et al.*, 2004; Woo *et al.*, 2008). Amide III (1220-1320 cm^{-1}) is related to C-N stretching and N-H bending, and is involved with the triple helical structure of collagen (Woo *et al.*, 2008). The spectra for ASC and PSC differed slightly, indicating slight differences in secondary structure of collagens (Wang *et al.*, 2007). Amide I and II absorptions were observed at 1651 and 1549 cm^{-1} for ASC and at 1655 and 1549 cm^{-1} for PSC, respectively. The shoulder of both collagens appearing at 1637 cm^{-1} could be attributed to the triple helix absorption of collagens (Petibois *et al.*, 2006). The triple helical structure of ASC was also confirmed from the absorption ratio between Amide III (1238 cm^{-1}) and 1454 cm^{-1} bands, which was approximately equal to 1.0 (Wang *et al.*, 2007). The ratio of PSC was about 1.2. The similar results have been reported in ASC and PSC from the skin of deep-sea redfish (Wang *et al.*, 2007). The differences in those ratio and Amide I band between ASC and PSC indicated some changes in helical structure of PSC. However, the triple helical structure was still predominant in PSC fraction when the skin material was limited to digestion by pepsin. Both collagens also exhibited absorptions at 1031, 1060 and 1081

cm^{-1}, which arise from the C-OH stretching vibrations of the carbohydrate moieties attached to the protein (Petibois *et al.*, 2006). The result suggested that the collagens might contain carbohydrates, which are attached to hydroxylysine residues of the polypeptide chain by O-glycosidic bonds. The presence of 2-O-α-D-glucosyl-O-β-D-galactosyl-hydroxylysine and of O-β-D-galactosyl-hydroxylysine has been reported (Burghagen, 1999). Glucosylgalactosylhydroxylysine was also found in the collagen from sea cucumber (*Stichopus japonicus*) (Saito *et al.*, 2002).

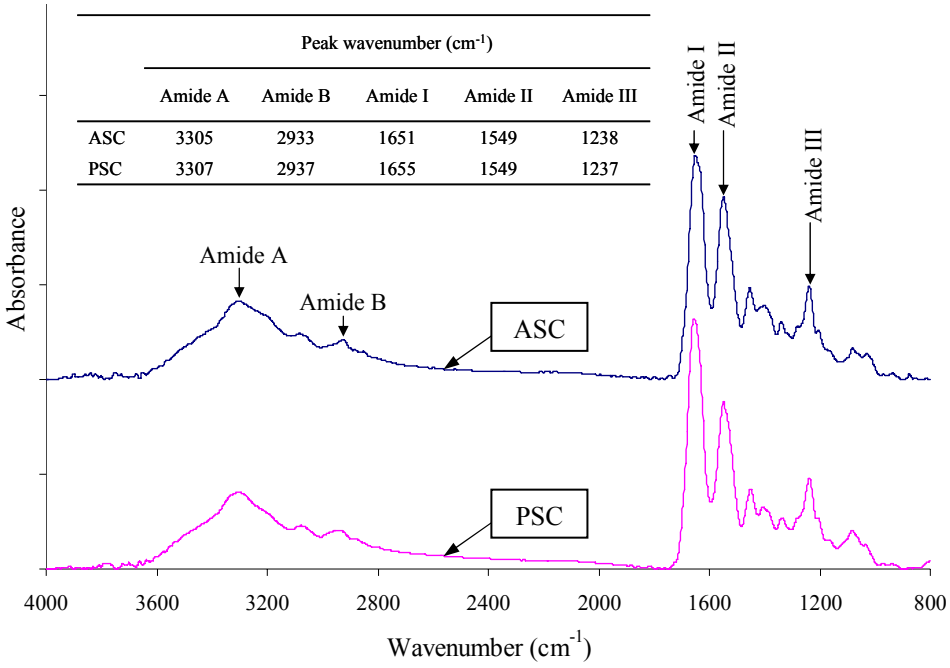

Figure 23. Fourier transform infrared spectra of acid solubilized collagen (ASC) and pepsin solubilized collagen (PSC) from the skin of unicorn leatherjacket. The inset shows wavenumber of different amide peaks of collagen.

6.4.6 Degradation patterns of skin collagen

The degradation patterns of ASC and PSC digested by chymotrypsin, in comparison with those of calf skin collagen type I, are shown in Figure 24. All collagens without preheating at 50 °C had slight degradation after being incubated with chymotrypsin at 25 °C for 15 min. Generally, the degradation patterns of ASC and PSC were similar. For collagens preheated at 50 °C for 30 min, both collagens were more susceptible to chymotryptic hydrolysis as evidence by the pronounced

degradation. No β, γ and α chains were retained in preheated ASC and PSC after chymotrypsin digestion. Coincidentally, peptide fragments with MW of 48-57, 40 and 36 kDa were produced. The band intensity of major components including α-, β- and γ-components of calf skin collagen decreased with the appearance of a 66 kDa peptide fragment and peptides with lower MW. These results revealed that cross-linked components (β- and γ-components) as well as α-components (α1 and α2) of preheated skin collagen from unicorn leatherjacket were easily digested by chymotrypsin than those of calf skin collagen under the condition used. Additionally, the results suggested that denatured collagen was readily cleaved by chymotrypsin whereas collagen in the native form (without preheating) was resistant to hydrolysis. Chymotrypsin catalyzes the hydrolysis of peptide bonds on the carboxyl side of hydrophobic amino acid residues, such as phenylalanine, tyrosine, tryptophan and leucine (Boyer, 2006). Due to the specificity of cleavage sites of chymotrypsin, the similar degradation patterns of ASC and PSC indicated the similarity in their primary structures, especially in terms of the sequence and the composition of amino acids, between both collagens.

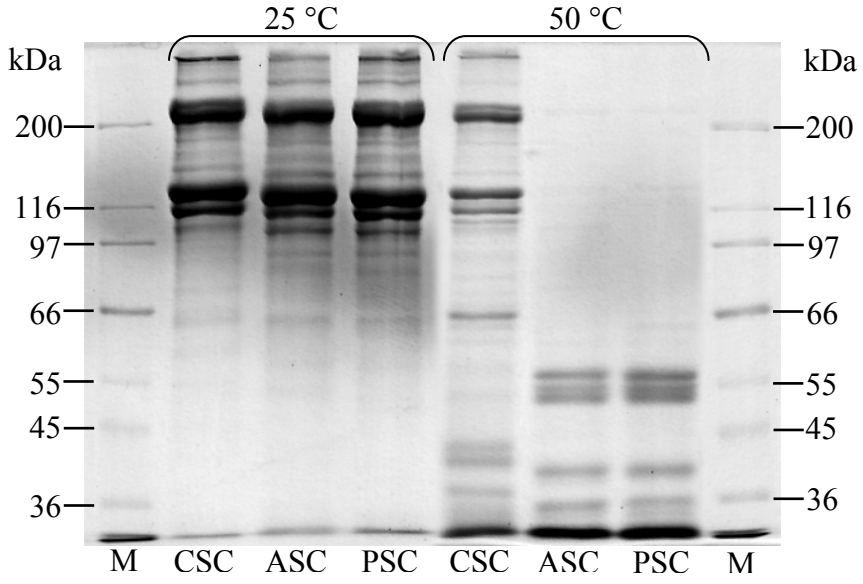

Figure 24. Degradation patterns of calf skin collagen (CSC), acid solubilzed collagen (ASC) and pepsin solubilized collagen (PSC) from the skin of unicorn leatherjacket. Collagen samples without and with pretreatment at 50 °C were digested by chymotrypsin at a level of 1 μg/mg collagen for 15 min at 25 °C. M denotes wide range MW protein markers.

6.4.7 ζ-potential of skin collagen

The ζ-potential of ASC and PSC at different pHs was shown in Figure 25. Generally, ζ-potential profiles of ASC and PSC were similar within pH range tested. The p*I* of ASC and PSC was estimated to be 6.29 and 6.37, respectively. The pH where the ζ-potential is zero corresponds to the p*I* of the protein (Ma *et al.*, 2009), in which a net electrical charge of zero at the surface is obtained. ASC and PSC had a net positive or negative charge when pH values were below and above their p*I*, respectively. At p*I*, the lowest solubility of skin collagens from brownstripe red snapper (Jongjareonrak *et al.*, 2005) and bigeye snapper (Kittiphattanabawon *et al.*, 2005) was noticeable. Net charges of protein molecules became zero at p*I* and hydrophobic–hydrophobic interaction increased, thereby promoting to the precipitation and aggregation (Jongjareonrak *et al.*, 2005). The result suggested that ASC and PSC possessed the similar surface properties as evidenced by the similarity in ζ-potential profiles.

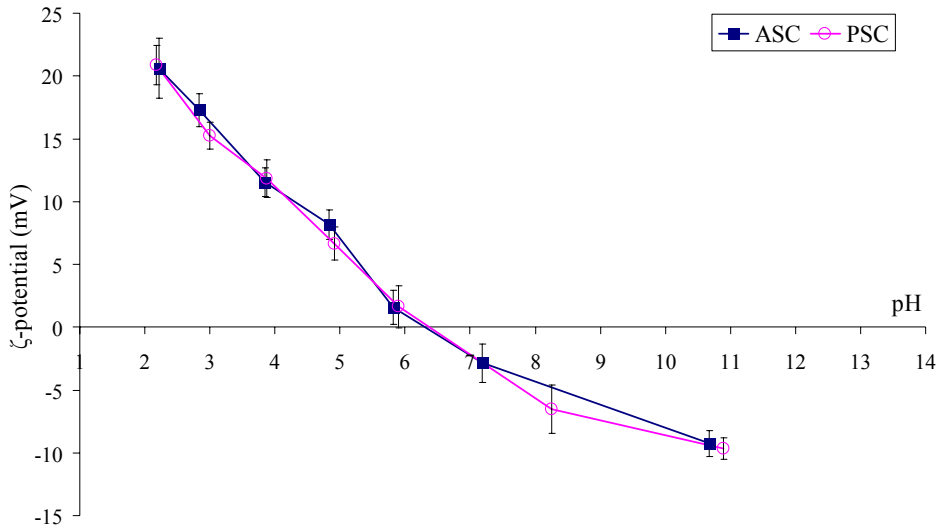

Figure 25. Influence of pH on the ζ-potential of acid solubilized collagen (ASC) and pepsin solubilized collagen (PSC) from the skin of unicorn leatherjacket.

6.5 Conclusion

Efficiency in collagen extraction from the skin of unicorn leatherjacket could be enhanced by 8 folds when pepsin from albacore tuna at 20 units/g of residual skin was used. PSC obtained exhibited similar molecular properties to ASC. ASC and PSC with predominant triple helical structure contained high content of imino acid. Both collagens were susceptible to chymotrypsin digestion after preheating at temperature higher than denaturation temperature. The skin of unicorn leather jacket could be a promising source for the collagen, particularly with the aid of fish pepsin.

CHAPTER 7

COLLAGENS FROM THE SKIN OF ARABESQUE GREENLING (*PLEUROGRAMMUS AZONUS*) SOLUBILIZED WITH THE AID OF ACETIC ACID AND PEPSIN FROM ALBACORE TUNA (*THUNNUS ALALUNGA*) STOMACH

7.1 Abstract

Acid solubilized collagen (ASC) from the skin of arabesque greenling was extracted with acetic acid. Pepsin solubilized collagen (PSC) was further extracted from the skin residue with the aid of pepsin from albacore tuna. The yields of ASC and PSC were 30.3 and 14.0% (dry wt.), respectively. Based on protein patterns and TOYOPEARL® CM-650M column chromatography, both collagens contained α- and β-chains as their major components and were characterized as type I collagen. A slightly lower molecular weight of α-chains of PSC was observed, in comparison with that of ASC. Both collagens contained glycine as a major amino acid and had imino acid content of 157-159 residues/1000 residues. The degradation induced by lysyl endopeptidase and V8-protease was more pronounced in PSC, compared with ASC. Transition temperatures of both collagens were in the range of 15.4-15.7 °C. Fourier transform infrared spectra revealed some differences in molecular order between ASC and PSC. Nevertheless, the triple-helical structure of PSC was still predominant. Based on ζ-potential, p*I* of ASC and PSC was estimated to be 6.38 and 6.31, respectively.

7.2 Introduction

Collagen is the fibrous protein of animal connective tissue, contributing to the unique physiological functions of tissues in skins, tendons, bones, cartilages, etc. and is associated with toughness in mammalian muscle (Foegeding *et al.*, 1996; Kittiphattanabawon *et al.*, 2010a; Muyonga *et al.*, 2004; Ogawa *et al.*, 2003; Yan *et al.*, 2008). Collagen is widely used in food, medicine, cosmetics and cell cultures and its consumption has been increasing along with the development of new industrial application (Woo *et al.*, 2008).

Nowadays, utilization of fish byproduct, especially skin, has received greater increasing attention due to the objection of collagen from mammal sources, particularly bovine and porcine origin due to the outbreak of mad cow disease and religion prohibition (Islam and Judaism), respectively (Duan *et al.*, 2009; Kittiphattanabawon *et al.*, 2010a; Nalinanon *et al.*, 2007; Nalinanon *et al.*, 2008b). As fish collagens have become the potential alternatives, collagens from several fish species such as ocellate puffer fish (Nagai *et al.*, 2002), black drum (Ogawa *et al.*, 2003), sheephead seabream (Ogawa *et al.*, 2003), brownstripe red snapper (Jongjareonrak *et al.*, 2005), Nile perch (Muyonga *et al.*, 2004), bigeye snapper (Benjakul *et al.*, 2010; Kittiphattanabawon *et al.*, 2005; Nalinanon *et al.*, 2007), channel catfish (Liu *et al.*, 2007), grass carp (Zhang *et al.*, 2007), deep-sea redfish (Wang *et al.*, 2007), yellowfin tuna (Woo *et al.*, 2008), walleye pollock (Yan *et al.*, 2008), threadfin bream (Nalinanon *et al.*, 2008b), carp (Duan *et al.*, 2009), brownbanded bamboo shark (Kittiphattanabawon *et al.*, 2010a) and blacktip shark (Kittiphattanabawon *et al.*, 2010b) have been extracted and characterized. The extraction of collagen from fish skin was generally started by the elimination of non-collagenous proteins prior to acid solubilization. The resulting collagen is referred to as “acid solubilized collagen, ASC”, in which a low yield is generally obtained. To tackle such a problem, pepsin capable of cleaving the peptides in the telopeptide region of collagen molecules has been used (Jongjareonrak *et al.*, 2005; Nagai *et al.*, 2002; Nalinanon *et al.*, 2007; Nalinanon *et al.*, 2008b). Zimmermann *et al.* (1970) found that 2 types of intermolecular bonds, side-to-side and end-to-end bond, in calf collagen can easily be cleaved by pepsin, whereas head-to-tail bond is relatively

pepsin resistant. After ASC extraction, the residues which represent the cross-linked molecules are further extracted in the presence of pepsin. The collagen obtained with pepsin treatment is referred to as "pepsin solubilized collagen, PSC". The use of pepsin as the aid of collagen extraction resulted in the increased yield (Jongjareonrak *et al.*, 2005; Kittiphattanabawon *et al.*, 2010a; Nagai *et al.*, 2002; Nalinanon *et al.*, 2007; Nalinanon *et al.*, 2008b). Generally, commercial pepsin used for collagen extraction is isolated from porcine stomach. Owing to the limitation of porcine pepsin mostly associated with the religious constraint, pepsins from fish origin including bigeye snapper (Nalinanon *et al.*, 2007), albacore tuna (Nalinanon *et al.*, 2008b) and tongol tuna (Benjakul *et al.*, 2010; Nalinanon *et al.*, 2008b) have been used as the potential aid for collagen extraction from fish skin. Those fish pepsins exhibited the comparable extraction efficacy to porcine counterpart. Therefore, fish pepsin could be used as a replacer of mammalian pepsin.

Arabesque greenling (*Pleurogrammus azonus*) is a species of mackerel. It is also known as "Okhotsk atka mackerel" and "hokke" in Japanese, in which the primary population of the fish is found off of the Sea of Okhotsk, Hokkaido, Japan. *P. azonus* is important in the fish-catches of Japan (Kishimura *et al.*, 2006) and its fillets are popular consumption in Japan. The skin produced could serve as the potential source of collagen or gelatin. However, no information on the isolation and characterization of collagen from the skin of arabesque greenling, which is an economically important species of Japan, has been reported. Therefore, the aim of this study was to isolate and characterize collagen from the skin of arabesque greenling without and with the aid of albacore tuna pepsin.

7.3 Materials and Methods

7.3.1 Chemicals

Bovine hemoglobin, β-mercaptoethanol (βME), type I collagen from calf skin, V8-protease from *Staphylococcus aureus* (EC 3.4.21.19) and lysyl endopeptidase from *Achromobacter lyticus* (EC 3.4.21.50) were purchased from Sigma Chemical Co. (St. Louis, MO, USA.). Trichloroacetic acid, disodium hydrogen

phosphate, sodium citrate, and acetic acid were obtained from Merck (Darmstadt, Germany). Coomassie Blue R-250 and *N,N,N′,N′*-tetramethyl ethylene diamine (TEMED) were procured from Bio-Rad Laboratories (Hercules, CA, USA). Sodium dodecyl sulfate (SDS) and bovine serum albumin were obtained from Fluka (Buchs, Switzerland). High-molecular-weight protein markers and TOYOPEARL® CM-650M were purchased from GE Healthcare UK Limited (Buckinghamshire, UK) and Tosoh Corporation (Tokyo, Japan), respectively.

7.3.2 Preparation of fish stomach and skin

Internal organs of albacore tuna (*Thunnus alalunga*) were obtained from Tropical Canning (Thailand) Public Co., Ltd., Songkhla, Thailand. The sample was packed in polyethylene bag, kept in ice with a solid/ice ratio of 1:2 (w/v) and transported to the Department of Food Technology, Prince of Songkla University, Hat Yai within 1 h. Upon the arrival, the pooled internal organs were excised and only stomach was collected and placed in polyethylene bag. The stomach sample was stored at -20 °C until use. The storage time was not longer than 2 months.

Arabesque greenling (*Pleurogrammus azonus*) were obtained after being off-loaded for 24 h from the dock in Hakodate, Hokkaido, Japan. The fish were kept in ice with a solid/ice ratio of 1:2 (w/v) and transported to the Laboratory of Marine Products and Food Science, Hokkaido University, Hakodate within 30 min. Upon the arrival, the skin was removed and washed with cold water (0-4 °C), drained and cut into small pieces (0.5 × 0.5 cm^2) using the scissor. The prepared skins were placed in polyethylene bag and stored at -20 °C until use. The storage time was less than 2 months.

7.3.3 Preparation of the extract from albacore tuna stomach

Frozen stomach was thawed using running water (26-28 °C) until the core temperature reached -2 to 0 °C. The sample was cut into pieces with a dimension of 0.5 × 0.5 cm^2. Sample was finely ground in liquid nitrogen using a National Model

MX-T2GN blender (Taipei, Taiwan). Sample was then lyophilized using a SCANVAC CoolSafe™ freeze-dryer (CoolSafe 55, ScanLaf A/S, Lynge, Denmark) and the dry powder was referred to as 'stomach powder'.

To extract pepsin, stomach powder was suspended in 20 mM sodium phosphate buffer (pH 7.0) at a ratio of 1:9 (w/v) and stirred continuously at 4 °C for 3 h. The suspension was centrifuged for 20 min at 4 °C at 20,000 ×*g* (H-2000B, Kokusan, Tokyo, Japan) to remove the tissue debris. The supernatant was collected and then adjusted to pH 4.0 with 1 M HCl. The mixture was allowed to stand at 4 °C for 30 min, followed by centrifugation at 20,000 ×*g* for 15 min at 4 °C. To fully activate pepsin, the supernatant was adjusted to pH 2.0 with 1 M HCl and gently stirred for 30 min at 4 °C prior to centrifugation at 20,000 ×*g* for 15 min at 4 °C. The supernatant containing activated pepsin was collected and referred to as "stomach extract".

7.3.4 Enzyme assay

Proteolytic activity of stomach extract was determined using hemoglobin as a substrate according to the method of Nalinanon *et al.* (2007) with some modifications. To initiate the reaction, 200 μL of stomach extract were added into the assay mixture containing 200 μL of 2% hemoglobin, 200 μL of distilled water and 600 μL of McIlvaine buffer (0.2 M sodium phosphate and 0.1 M sodium citrate, pH 2). Appropriate dilution was made to ensure that the amount of enzyme was not excessive for available substrate in the assay system. The reaction was conducted at pH 2.0 and 50 °C for 20 min. To terminate enzymatic reaction, 200 μL of 50% (w/v) trichloroacetic acid (TCA) were added. Unhydrolyzed protein substrate was allowed to precipitate for 1 h at 4 °C, followed by centrifuging at 15,000 ×*g* for 10 min using a KUBOTA 3630 centrifuge (SiGMA Laborzentrifugen, Osterode am Harz, Germany). The oligopeptide content in the supernatant was measured at 280 nm. One unit of activity was defined as the amount causing an increase of 1.0 in absorbance at 280 nm per min (A_{280} min^{-1}). A blank was run in the same manner,

except that the enzyme was added into the reaction mixture after the addition of 50% (w/v) TCA.

7.3.5 Preparation of skin collagen

Collagens from the skin of arabesque greenling were isolated following the method of Nalinanon *et al.* (2007) and Jongjareonrak *et al.* (2005) with some modifications. All procedures were performed at 4 °C. To remove non-collagenous proteins, the skin was mixed with 0.1 M NaOH using a sample/alkaline solution ratio of 1:10 (w/v). The mixture was stirred continuously for 6 h. The alkaline solution was changed every 2 h. Then, the alkaline treated skin was washed with cold water until neutral or faintly basic pHs of wash water were obtained. The treated skin was then defatted with 10% (v/v) 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 20 volumes of cold water for 3 times.

To extract the collagen, the prepared skin was soaked in 0.5 M acetic acid with a sample/solution ratio of 1:30 (w/v) for 24 h with gentle stirring using LABO-STIRRER model LR-51B stirrer (Yamato Scientific Co., Ltd., Tokyo, Japan). The mixture was then centrifuged at 20,000 ×*g* for 1 h. The supernatant was collected and kept at 4 °C. The precipitate was re-extracted in the same manner. Both supernatants obtained were combined and added with NaCl to obtain a final concentration of 2.6 M in 0.05 M Tris–HCl (pH 7.5). The resultant precipitate was collected by centrifuging at 20,000 ×*g* for 1 h. The pellet was dissolved in 10 volumes of 0.5 M acetic acid. The solution obtained was dialyzed against 10 volumes of 0.1 M acetic acid in a dialysis bag with a molecular weight cut-off of 14 kDa for 12 h with the change of dialysis solution every 4 h. Subsequently, the solution was dialyzed with 30 volumes of distilled water. The changes of dialysis water were performed until neutral pH was obtained. The dialysate was freeze-dried and referred to as ‘acid solubilized collagen, ASC’.

Undissolved residue obtained after acid extraction was suspended in 3 volumes of 0.5 M acetic acid in the presence of albacore tuna pepsin (150 units/g residual dry skin). The mixture was gently stirred for 24 h, followed by centrifugation

at 20,000 ×*g* for 1 h. Then, the supernatant was immediately precipitated by the addition of NaCl to a final concentration of 2.6 M in 0.05 M Tris–HCl (pH 7.5). The mixture was allowed to stand for 1 h for pepsin inactivation. The resultant precipitate was collected by centrifugation at 20,000 ×*g* for 1 h and dissolved in 10 volumes of 0.5 M acetic acid. The solution was dialyzed and freeze-dried in the same manner as for ASC preparation. Dry matter was referred to as 'pepsin solubilized collagen, PSC'. ASC and PSC were subjected to analyses. The yield of collagen was calculated based on the weight of lyophilized resulting collagen in comparison with that of dry defatted skin.

7.3.6 Characterization of collagen

7.3.6.1 Hydroxyproline content

Collagens were analyzed for hydroxyproline content after hydrolysis of the sample in 6 M HCl at 110 °C for 24 h, using the colorimetric method as described by Nalinanon *et al.* (2007). The Hydroxyproline content was calculated and expressed as mg/g of lyophilized sample.

7.3.6.2 UV absorption spectra

Collagens were dissolved in 0.5 M acetic acid to obtain a concentration of 1 mg/mL. The solution was placed into a quartz cell with a path length of 1 cm. UV absorption spectra of collagens were measured using a double-beam spectrophotometer (UV-1700, Shimadzu, Kyoto, Japan). Prior to measurement, the base line was set with 0.5 M acetic acid. The spectra were obtained by scanning the wavelength in the range of 190–350 nm with a scan speed of 50 nm/min at room temperature.

7.3.6.3 Amino acid analysis

Collagens 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).

7.3.6.4 SDS–polyacrylamide gel electrophoresis (SDS–PAGE)

SDS–PAGE was performed by the method of Laemmli (1970). The samples were mixed with 5% (w/v) SDS and heated at 85 °C for 1 h. The mixtures were then centrifuged at 8,500 ×*g* for 5 min to remove undissolved debris. Solubilized samples were mixed at 1:1 (v/v) ratio with the sample buffer (0.5 M Tris–HCl, pH 6.8, containing 4% (w/v) SDS, 20% (v/v) glycerol) in the presence and absence of 10% (v/v) β-ME. Samples (15 μg protein) were loaded onto a polyacrylamide gel made of 7.5% or 5% separating gel and 4% stacking gel and subjected to electrophoresis at a constant current of 15 mA/gel, using a Mini-PROTEAN II unit (Bio-Rad Laboratories Inc., Richmond, CA, USA). After electrophoresis, gels were fixed with a mixture of 50% (v/v) methanol and 10% (v/v) acetic acid for 30 min, followed by staining with 0.05% (w/v) Coomassie Blue R-250 in 15% (v/v) methanol and 5% (v/v) acetic acid for 1 h. Finally, they were destained with the mixture of 30% (v/v) methanol and 10% (v/v) acetic acid for 45 min. Gels were imaged using a Canon image scanner (CanoScan LiDE 25, Canon Inc., Tokyo, Japan) and band intensities were quantified with the public domain digital analysis software, ImageJ (ImageJ 1.42q, National Institutes of Health, Bethesda, MD, USA). High-molecular-weight protein markers (GE Healthcare UK Limited, Buckinghamshire, UK) were used to estimate the molecular weight of proteins. Type I collagen from calf skin was used as a standard.

7.3.6.5 TOYOPEARL® CM-650M column chromatography

TOYOPEARL® CM-650M column chromatography was carried out according to the method of Kittiphattanabawon *et al.* (2010a) and Nagai *et al.* (2008) with some modifications. Collagen samples (20 mg) were dissolved in 5 mL of starting buffer (50 mM sodium acetate buffer, pH 4.8 containing 2 M urea) and incubated at 60 °C for 30 min. The mixtures were centrifuged at 20,000 ×*g* at room temperature (25–26 °C) for 30 min. The supernatants were applied onto a TOYOPEARL® CM-650M column (1.0 × 5.0 cm) previously equilibrated with 10

volumes of the starting buffer at a flow rate of 60 mL/h. After loading, the unbound proteins were washed by the same buffer until A_{230} was less than 0.05. Elution was achieved with a linear gradient of 0–0.2 M NaCl in the same buffer at a flow rate of 30 mL/h with a total volume of 100 mL. The eluant was monitored at 230 nm and 2-mL fractions were collected. The selected fractions were subjected to SDS–PAGE using 7.5% separating gel and 4% stacking gel as previously described.

7.3.6.6 Differential scanning calorimetry (DSC)

Prior to analysis, dry collagen samples were rehydrated with distilled water using a solid/solution ratio of 1:40 (w/v). The mixture was allowed to stand for 2 days at 4 °C. Differential scanning calorimetry (DSC) was performed using a differential scanning colorimeter (Model DSC 7, Norwalk, Connecticut, USA). Temperature calibration was done using the indium thermogram. The collagen solutions (5–10 mg) were accurately weighed into aluminium pans and sealed. The samples were scanned at 1 °C min^{-1} over the range of 5–50 °C using dry ice as the cooling medium. An empty pan was used as the reference. The maximum transition temperature (T_m) was estimated from the DSC thermogram.

7.3.6.7 Fourier transform infrared (FTIR) spectroscopy

Spectra of collagens from the skin of arabesque greenling were obtained by using a Bruker Model EQUINOX 55 FTIR spectrometer (Bruker, Ettlingen, Germany) equipped with a deuterated l-alanine tri-glycine sulfate (DLATGS) detector. The Horizontal Attenuated Total Reflectance Accessory (HATR) was mounted into the sample compartment. The internal reflection crystal (Pike Technologies, Madison, WI, USA), which was made of zinc selenide, had a 45° angle of incidence to the IR beam. Spectra were acquired at a resolution of 4 cm^{-1} and the measurement range was 4000–800 cm^{-1} (mid-IR region) at room temperature. Automatic signals were collected in 32 scans at a resolution of 4 cm^{-1} and were ratioed against a background spectrum recorded from the clean, empty cell at 25 °C. Analysis of spectral data was carried out using the OPUS 3.0 data collection software program (Bruker, Ettlingen, Germany).

7.3.6.8 Peptide mapping

Peptide mapping of collagen samples was performed according to the method of Jongjareonrak *et al.* (2005) with a slight modification. The freeze-dried samples (2 mg) were dissolved in 0.5 mL of 0.1 M sodium phosphate, pH 7.2 containing 0.5% (w/v) SDS. After the addition of 20 μL of the same buffer containing 1 μg of *S. aureus* V8 protease or 0.1 μg of lysyl endopeptidase from *A. lyticus* to collagen solutions, the reaction mixture was incubated at 37 °C for 60 min. The reaction was terminated by subjecting the reaction mixture to boiling water for 3 min. Peptides generated by the protease digestion were separated by SDS–PAGE using 7.5% gel. Peptide mapping of calf skin collagen was conducted in the same manner and the peptide maps were compared.

7.3.6.9 Measurement of zeta (ζ) potential

Collagen samples were dissolved in 0.5 M acetic acid at a concentration of 0.5 mg/mL. The mixture was stirred at 4 °C for 12 h. The ζ-potential of each sample (20 mL) was measured using a Zeta potential analyzer (ZetaPALS, Brookhaven Instruments Co., Holtsville, New York, USA). ζ-potential of samples adjusted to different pHs with 1.0 M nitric acid or 1.0 M KOH using an Autotitrator (BI-ZTU, Brookhaven Instruments Co., Holtsville, New York, USA) was determined. The isoelectric point (p*I*) was estimated from pH rendering zero ζ-potential.

7.3.7 Statistical analysis

All data were subjected to Analysis of Variance (ANOVA) and the differences between means were evaluated by Duncan's multiple range test. For pair comparison, T-test was used. SPSS statistic program (SPSS 11.0 for Windows, SPSS Inc., Chicago, IL, USA) was used for data analysis.

7.4 Results and Discussion

7.4.1 Yield and purity of collagens

The yield of ASC and PSC from the skin of arabesque greenling was 30.3 and 14.0% (dry wt.), respectively (Table 17). The accumulative yield of collagen (44.3%) was obtained when albacore tuna pepsin was used to extract the residual collagen in skin after acid solubilization process. Yield of collagen extracted from brownbanded bamboo shark (Kittiphattanabawon *et al.*, 2010a), black drum and sheepshead seabream (Ogawa *et al.*, 2003) increased with the aid pepsin from porcine stomach. Ogawa *et al.* (2003) reported that the yields of ASCs from black drum (*Pogonias cromis*) and sheepshead seabream (*Archosargus probatocephalus*) were estimated at 2.3 and 2.6% (dry wt.), and the yields of PSCs were 15.8 and 29.3% (dry wt.), respectively. The yield of ASC and PSC from the skin of brownbanded bamboo shark (*Chiloscyllium punctatum*) was 24.66 and 23.07% (dry wt.), respectively (Kittiphattanabawon *et al.*, 2010a). The result indicated that the insoluble collagen in the skin of arabesque greenling could not be completely solubilized with 0.5 M acetic acid. However, it was readily solubilized by the limited hydrolysis with pepsin. The swollen skin after acid extraction might has a loosened matrix, leading to the ease of pepsin to cleave the telopeptides (Kittiphattanabawon *et al.*, 2010a; Nalinanon *et al.*, 2007). Therefore, albacore tuna pepsin was shown as the effective aid for collagen extraction from the skin of arabesque greenling. Collagen extraction with the increased yield was achieved for the skin of bigeye snapper (Benjakul *et al.*, 2010; Nalinanon *et al.*, 2007) and threadfin bream (Nalinanon *et al.*, 2008b) when fish pepsin was used as the extraction aid.

Table 17. Yield and characteristics of ASC and PSC from the skin of arabesque greenling*.

	ASC	PSC#
Yield (% dry wt.)	30.3 ± 0.3	14.0 ± 0.2
Hydroxyproline content (mg/g dry wt.)	66.7 ± 0.8 NS	66.5 ± 0.6 NS
UV absorption peak (nm)	234 ± 0.2 NS	234 ± 0.3 NS
T_m (°C)	15.7 ± 0.3 NS	15.4 ± 0.2 NS

* Mean ± SD from triplicate determinations.

NS No significant difference ($P > 0.05$).

PSC was extracted from the residue of ASC extraction.

Generally, collagen contains no tryptophan and has a low content of tyrosine and phenylalanine, which could absorb UV-light at 280 nm. When the absorbance spectra were determined for both ASC and PSC, the distinct absorption peak of both ASC and PSC was observed at 234 nm. This might be attributed to the absorption of peptide bonds in the polypeptides chains of collagen. No absorption peak was detected at 280 nm (data not shown). The results suggested that both collagens contained low content of non-collagenous proteins. This absorption characteristic was in accordance with that of the collagens from the skin of walleye pollock (Yan *et al.*, 2008), Nile tilapia (Zeng *et al.*, 2009), carp (Duan *et al.*, 2009), shark (Kittiphattanabawon *et al.*, 2010a) and channel catfish (Liu *et al.*, 2007). ASC and PSC had similar hydroxyproline content (66.7 and 66.5 mg/g, respectively) ($P > 0.05$), suggesting the similarity in purity (Table 17). It was also noted that non-collagenous proteins could be removed effectively from the skin prior to collagen extraction.

7.4.2 Protein patterns and subunit compositions of collagens

Protein patterns of ASC and PSC from the skin of arabesque greenling, under reducing and non-reducing conditions, are shown in Figure 26. For each collagen, the similar protein patterns analyzed under both conditions were observed. The result indicated that both ASC and PSC contained no interchain disulfide bonds.

Both collagens consisted of two different α-chains (α1 and α2) and their cross-linked components, dimer and trimer refered to as β and γ, respectively. The results were in accordance with those found in collagen extracted from the skin of bigeye snapper (Kittiphattanabawon *et al.*, 2005; Nalinanon *et al.*, 2007), brownstripe red snapper (Jongjareonrak *et al.*, 2005), carp (Duan *et al.*, 2009), channel catfish (Liu *et al.*, 2007), threadfin bream (Nalinanon *et al.*, 2008b), black drum (Ogawa *et al.*, 2003) and sheepshead seabream (Ogawa *et al.*, 2003). Since α3 component had the same MW with α1 chain, both chains could migrate electrophoretically to the same position (Ogawa *et al.*, 2003). Therefore, both collagens were most likely the heterotrimer with the chain composition of $(\alpha1)_2\alpha2$ or α1α2α3. This observation was in accordance with those previously reported for skin collagen from black drum (Ogawa *et al.*, 2003) and sheepshead seabream (Ogawa *et al.*, 2003). The lower band intensity of high molecular weight (MW) cross-linked components and γ-chain was noticeable in PSC, compared with that of ASC. It was suggested that those components were converted to lower MW components such as α- and β -chains by albacore tuna pepsin digestion. Furthermore, proteins with MW of 141 and 54 kDa were also found in PSC. As a result, more extractable collagens could be obtained when pepsin treatment was applied. Nevertheless, the slight difference in relative mobility between different collagens was observed. Type I collagen from calf skin consisted of α1 and α2 chains with MW of 128 and 116 kDa, respectively. The MW of α1 and α2 chains of ASC was estimated to be 121 and 111 kDa, respectively. For PSC, MW of α1 and α2 chains was 115 and 106 kDa, respectively. MW of calf skin collagen was slightly higher than that of collagens from the skin of arabesque greenling. A slightly lower MW of α-chains of PSC was obtained, compared with that of ASC, suggesting that PSC might undergo the partial cleavage at telopeptide regions by pepsin treatment. The similar result was previously found in the collagen extraction from the skin of bigeye snapper using pepsin from bigeye snapper stomach (Nalinanon *et al.*, 2007). Drake *et al.* (1966) reported that some of the telopeptides of calf skin tropocollagen are susceptible to pepsin hydrolysis and intramolecular cross-links are broken on pepsin digestion.

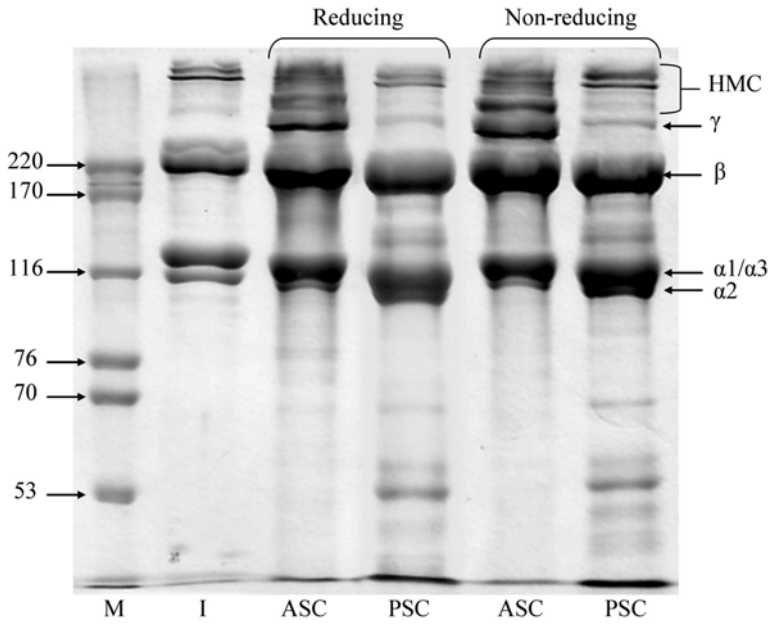

Figure 26. SDS-PAGE patterns of ASC and PSC from the skin of arabesque greenling under reducing and non-reducing conditions. M, I and HMC denote high-molecular-weight protein markers, type I collagen from calf skin and high MW cross-linked components, respectively.

The elution profiles of ASC and PSC on the TOYOPEARL® CM-650M column after being denatured by heat treatment in the presence of 2 M urea are shown in Figure 27. The differences in chromatograms of both collagens were observed, in which the peaks and collagen components were found at different retention times. The chromatographic fractions indicated by numbers were subjected to SDS-PAGE. For ASC, the first peaks (fraction no. 19) contained α1 chain, while the second peak (fraction no. 24) consisted of both α1 and α2 chains at a ratio of 2:1, most likely type I collagen (Figure 27A). Also, this fraction had β12 and γ chains. For PSC, the first small peak at fraction no. 17 contained no major components of collagen (Figure 27B). Only α1-chain was observed in the fraction no. 19. Fraction no. 23 consisted of α1-chain, β11 and β12 chains. Apart from β-chain, fraction no. 27 contained both α1 and α2 chains at a ratio of 2:1. It was noted that higher γ-band intensity was obtained in ASC, compared with that of PSC. This suggested the conversion of γ-chain by pepsin to the smaller MW components. β11-dimer of ASC was found in the fraction no. 17-21, while that component was concentrated in

fraction no. 23 of PSC. The different elution profiles of ASC and PSC indicated that the primary structure of collagen from the skin of arabesque greenling was modified to some degree by albacore tuna pepsin digestion. β12/β22 dimer of ASC and PSC was the major component observed in the fraction no. 24-30, which had the different band intensities. High MW component and γ-chain were also detected in those fractions. It was suggested that some of α2 component might either dimerize covalently into β-component and form β12- or β22-dimer or polymerize into higher MW components. As a result, much lower band intensity of α2 chain was detected on SDS-PAGE and band intensity ratios of α1/α2 more than 2-fold were observed (Figure 26). Similar results have been reported for collagen from the skin of brownbanded bamboo shark (Kittiphattanabawon *et al.*, 2010a) and blacktip shark (Kittiphattanabawon *et al.*, 2010b). Therefore, based on electrophoretic patterns and elution profiles, ASC and PSC from the skin of arabesque greenling were most likely classified to be type I collagen.

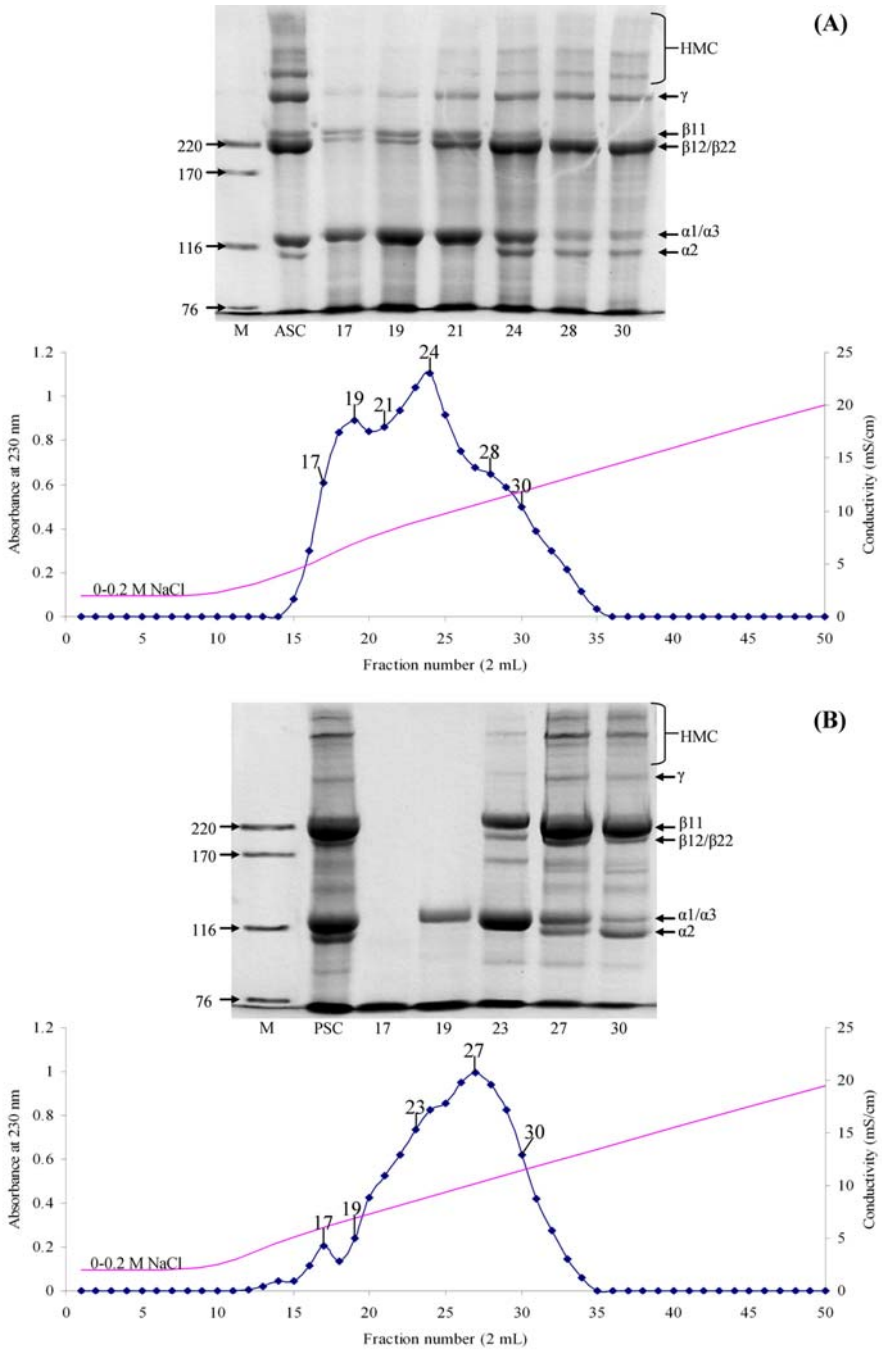

Figure 27. Elution profiles of ASC (A) and PSC (B) from the skin of arabesque greenling on the TOYOPEARL® CM-650M ion-exchange column. The fractions indicated by numbers were examined by SDS–PAGE using 5% separating gel and 4% stacking gel. M and HMC denote high-molecular-weight protein markers and high MW cross-linked components, respectively.

7.4.3 Amino acid composition

The amino acid composition of ASC and PSC is expressed as residues per 1000 total amino acid residues as shown in Table 18. Generally, ASC and PSC extracted from the skin of arabesque greenling had similar amino acid profile. The collagens were rich in glycine, proline and alanine, and contained no cysteine and tryptophan. They contained relatively low content of tyrosine and histidine. Similar result was reported for type I collagens from other fish species such as black drum (Ogawa *et al.*, 2003), sheephead seabream (Ogawa *et al.*, 2003), bigeye snapper (Nalinanon *et al.*, 2007) and brownstripe red snapper (Jongjareonrak *et al.*, 2005). Both ASC and PSC consisted of proline and hydroxyproline, which are unique amino acids in collagen (Jongjareonrak *et al.*, 2005). The imino acid contents (proline and hydroxyproline) of ASC and PSC from the skin of arabesque greenling were 159 and 157 residues/1000 residues, respectively. Relatively low imino acid content of the skin collagens from arabesque greenling, a cold water species, was in accordance with those of other cold water fish such as cod (154 residues/1000 residues) (Duan *et al.*, 2009) and deep-sea red fish (160-165 residues/1000 residues) (Wang *et al.*, 2007). However, imino acid contents of both ASC and PSC were much lower than those reported for collagen from the skin of carp (Duan *et al.*, 2009), black drum and sheephead (Ogawa *et al.*, 2003), brownstripe red snapper (Jongjareonrak *et al.*, 2005) and bigeye snapper (Kittiphattanabawon *et al.*, 2005), which contained imino acids ranging from 190 to 221 residues per 1000 residues. The difference in imino acid content among animals was associated with the varying living environments, particularly habitat temperature (Foegeding *et al.*, 1996; Jongjareonrak *et al.*, 2005; Kittiphattanabawon *et al.*, 2010a). Imino acids generally contribute to the stability of helix structure of collagen (Jongjareonrak *et al.*, 2005). The imino acids content was also reported to have a major influence on thermal stability of collagen (Kittiphattanabawon *et al.*, 2010a). Pyrrolidine rings of proline and hydroxyproline impose restrictions on the conformation of the polypeptide chain and help to strengthen the triple helix (Bae *et al.*, 2008). Hydroxylysine of 6-7 residues per 1000 residues was also found in both collagens, suggesting the partial cross-linking of collagen via covalent bond (Mechanic *et al.*, 1987).

Table 18. Amino acid composition of ASC and PSC from the skin of arabesque greenling (residues/1000 residues)

Amino acid	**ASC**	**PSC**
Alanine	103	99
Arginine	54	53
Aspartic acid/asparagine	52	55
Cysteine	0	0
Glutamic acid/glutamine	78	80
Glycine	344	335
Histidine	7	7
Isoleucine	9	11
Leucine	20	24
Lysine	26	28
Hydroxylysine	6	7
Methionine	15	14
Phenylalanine	13	13
Hydroxyproline	57	58
Proline	102	99
Serine	69	67
Threonine	23	25
Tyrosine	3	3
Tryptophan	0	0
Valine	19	21
Total	**1000**	**1000**
Imino acids *	159	157

* Imino acids include proline and hydroxyproline

7.4.4 Peptide mapping

The peptide maps of ASC and PSC from the skin of arabesque greenling digested by lysyl endopeptidase and V8-protease, in comparison with those of type I collagen from calf skin, are shown in Figure 28. All collagens were susceptible to digestions by both enzymes. Degree of hydrolysis varied with types of collagen and enzyme used. Calf skin collagen was generally resistant to V8-protease hydrolysis but was susceptible to hydrolysis by lysyl endopeptidase. The degradation patterns of calf skin collagen were different from those of collagens from the skin of arabesque greenling. Lysyl endopeptidase is a serine endoprotease which hydrolyzes peptide bonds at the carboxyl side of lysyl residues (Jekel *et al.*, 1983). V8-protease

exhibits a high degree of specificity for glutamic acid and aspartic acid residues of peptides and proteins (Vercaigne-Marko *et al.*, 2000). Due to the specificity of cleavage sites of those enzymes, the different degradation peptides between calf skin collagen and arabesque greenling collagens were generated. This indicated the difference in their primary structures, especially in terms of the sequence and the composition of amino acids, between collagens from different sources. When comparing the degradation pattern between ASC and PSC, it was found that the latter was more susceptible to hydrolysis, compared with the former. This was evidenced by the decreases in band intensity of high MW component and the appearance of low MW peptides, especially at the dye front. The cleavage of telopeptide region by pepsin might facilitate the changes in configurations which favored the hydrolysis by both lysyl endopeptidase and V8-protease. No β, γ and α chains were observed after lysyl endopeptidase digestion, while those components were still retained to some extent after V8-protease hydrolysis. Generally, band intensity of major components including α-, β- and γ-components of both collagens decreased after digestion with both enzymes.

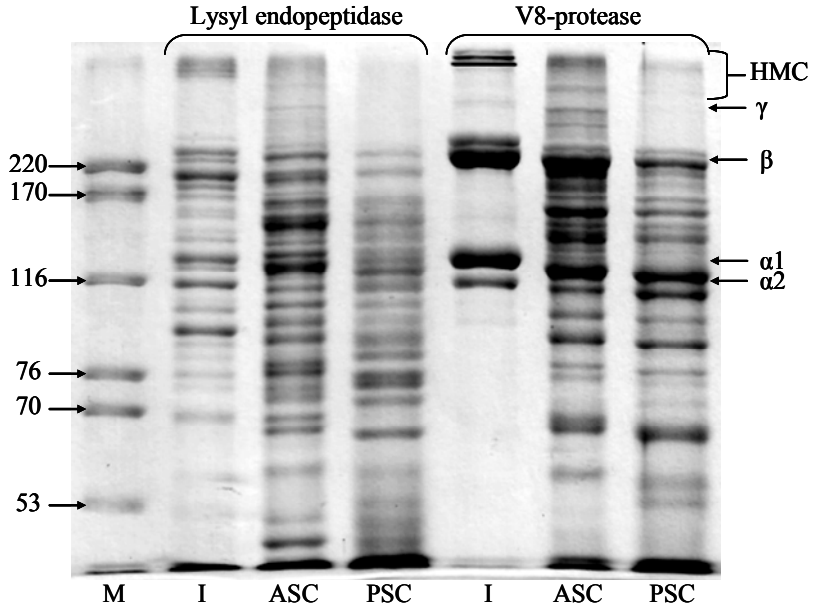

Figure 28. Peptide maps of ASC and PSC from the skin of arabesque greenling digested by lysyl endopeptidase or V8-protease. M, I and HMC denote high-molecular-weight protein markers, type I collagen from calf skin and high MW cross-linked components, respectively.

7.4.5 Thermal stability

Maximal transition temperatures (T_m) of ASC and PSC from the skin of arabesque greenling were 15.7 and 15.4 °C, respectively (Table 17). T_m of ASC was similar to that of PSC, suggesting no differences in the denaturation temperature between both collagens. Therefore, pepsin digestion did not affect the denaturation temperature of PSC, though a slight decrease in MW was obtained (Figure 26). T_{max} of collagens from the skin of arabesque greenling was in accordance with that of cold water fish species such as cod (15 °C) (Duan *et al.*, 2009) and deep-sea redfish (16.1 °C) (Wang *et al.*, 2007). Denaturation temperatures of skin collagen from several cold and temperate water fish including carp (28 °C) (Duan *et al.*, 2009), grass carp (28.4 °C) (Zhang *et al.*, 2007), ocellate puffer fish (28 °C) (Nagai *et al.*, 2002), tiger puffer fish (28.4 °C) (Bae *et al.*, 2008), Japanese seabass (26.5 °C), chub mackerel (25.6 °C) and bullhead shark (25 °C) (Nagai and Suzuki, 2000) have been reported. Denaturation temperatures of collagens from the skin of arabesque greenling were much lower than those of subtropical and tropical fish such as black drum (34.2 °C) (Ogawa *et al.*, 2003), sheephead seabream (34 °C) (Ogawa *et al.*, 2003), bigeye snapper (32.5 °C) (Nalinanon *et al.*, 2007) and brownstripe red snapper (30.5 °C) (Jongjareonrak *et al.*, 2005). The differences in denaturation temperature of collagen from different sources might be governed by different contents of imino acids (proline and hydroxyproline) (Jongjareonrak *et al.*, 2005; Kittiphattanabawon *et al.*, 2010a; Zhang *et al.*, 2007). The thermal stability of collagen is associated with the restriction of the secondary structure of the polypeptide chain governed by the pyrrolidine rings of proline and hydroxyproline and partially by the hydrogen bonding through the hydroxyl group of hydroxyproline (Benjakul *et al.*, 2010). Furthermore, imino acid content of fish collagen and their thermal stability are associated with water temperature of their normal habitat (Foegeding *et al.*, 1996).

7.4.6 FTIR spectra

FTIR spectra of ASC and PSC from the skin of arabesque greenling are shown in Figure 29. The collagens from the skin of arabesque greenling exhibited

FTIR spectra similar to those found in other fish skin collagens (Duan *et al.*, 2009; Kittiphattanabawon *et al.*, 2010a; Kittiphattanabawon *et al.*, 2010b; Liu *et al.*, 2007; Muyonga *et al.*, 2004; Wang *et al.*, 2007; Woo *et al.*, 2008), in which the absorption bands were situated in the amide band region including amides A, B, I, II and III. FTIR spectra of ASC and PSC were slightly different, indicating slight differences in the secondary structure (Wang *et al.*, 2007) and functional groups of collagens. The amide A band of ASC and PSC were found at 3298 and 3296 cm^{-1}, respectively. This band is generally associated with the N–H stretching vibration and shows the existence of hydrogen bonds (Kittiphattanabawon *et al.*, 2010a; Woo *et al.*, 2008). The lower wavenumber of amide A band of PSC indicated that more N-H group was involved in hydrogen bond (Wang *et al.*, 2007). Amide B band of both collagens was observed at 2924-2925 cm^{-1}, which was in accordance with that reported by Nagai et al. (Nagai *et al.*, 2008) and Kittiphattanabawon *et al.* (2010a). The amide I and amide II bands of both ASC and PSC were observed at the wavenumber of 1640-1641 cm^{-1} and 1531-1537 cm^{-1}, respectively. The amide I band, with characteristic frequencies in the range of 1600-1700 cm^{-1}, is mainly associated with the stretching vibrations of the carbonyl groups (C=O bond) along the polypeptide backbone and is a sensitive marker of polypeptide secondary structure (Kittiphattanabawon *et al.*, 2010b; Payne and Veis, 1988; Wang *et al.*, 2007). The shoulder of both collagens appearing at 1637 cm^{-1} could be attributed to the triple helix absorption of collagens (Petibois *et al.*, 2006). Amide II (~1550 cm^{-1}) is associated with N-H bending coupled with C-N stretching (Muyonga *et al.*, 2004; Woo *et al.*, 2008). Amide III band of ASC and PSC was observed at 1234 cm^{-1}, which was related to C-N stretching and N–H in plane bending from amide linkages as well as absorptions arising from wagging vibrations from CH_2 groups from the glycine backbone and proline side-chains (Mohd Nasir *et al.*, 2006), involved in triple-helical structure of collagen (Muyonga *et al.*, 2004; Wang *et al.*, 2007; Woo *et al.*, 2008). The triple helical structure of ASC and PSC was also confirmed from the absorption ratio between Amide III (1234 cm^{-1}) and 1450 cm^{-1} bands, which was approximately equal to 1.0 (Kittiphattanabawon *et al.*, 2010a; Plepis *et al.*, 1996; Wang *et al.*, 2007). However, a slight difference in this ratio between ASC (1.1) and PSC (1.3) was found. The result suggested that pepsin might affect the triple-helical structure of collagen to some degree. The similar results have

been reported in ASC and PSC from the skin of deep-sea redfish (Wang *et al.*, 2007). The differences in those ratios and wavenumbers of amide bands between ASC and PSC indicated some differences in the molecular structure between ASC and PSC.

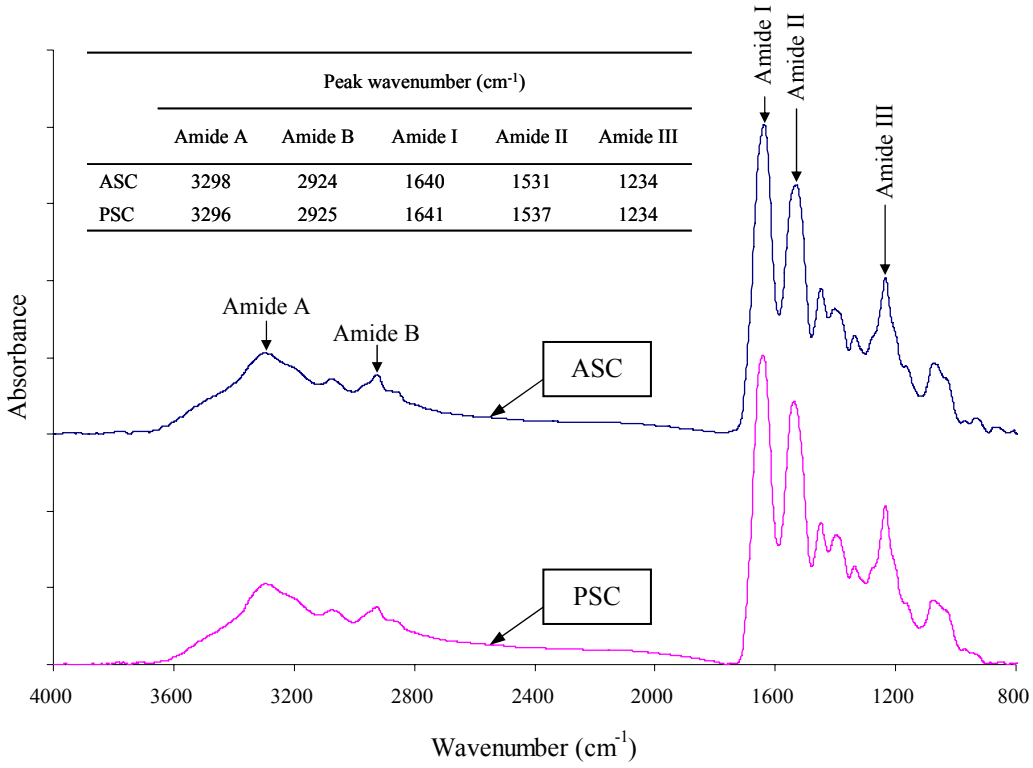

Figure 29. FTIR spectra of ASC and PSC from the skin of arabesque greenling. The inset shows wavenumber of different amide peaks of collagen.

7.4.7 ζ-potential of skin collagens

The ζ-potential of ASC and PSC at different pHs is shown in Figure 30. Generally, ζ-potential profiles of ASC and PSC were similar within pH range tested. The p*I* of ASC and PSC was estimated to be 6.38 and 6.31, respectively. The pH where the ζ-potential is zero corresponds to the p*I* of the protein (Ma *et al.*, 2009), in which a net electrical charge of zero at the surface is obtained. At p*I* of proteins, hydrophobic–hydrophobic interaction increased, thereby promoting the precipitation and aggregation of protein molecules (Jongjareonrak *et al.*, 2005). ASC and PSC had a net positive or negative charge when pH values were below and above their p*I*, respectively. Both collagens had p*I* in a slight acidic pH. This might be due to higher

content of acidic amino acids, aspartic acid and glutamic acid, compared to basic amino acids, including histidine, lysine and arginine. The similarity in ζ-potential profiles of ASC and PSC indicated that partial removal of telopeptides in PSC by the albacore tuna pepsin did not significantly alter surface charge of collagen.

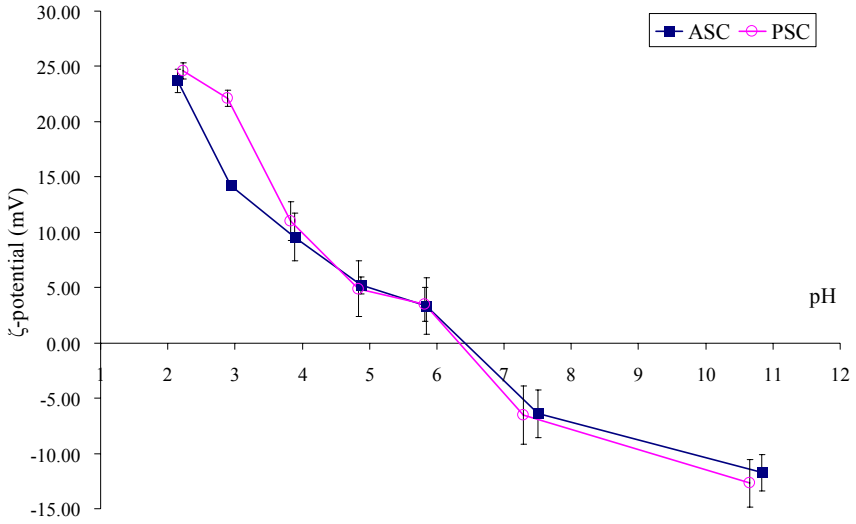

Figure 30. Zeta (ζ) potential of ASC and PSC from the skin of arabesque greenling at different pHs. Bars represent the standard deviation from triplicate determinations.

7.5 Conclusion

Collagen from the skin of arabesque greenling could be easily extracted by acetic acid. Use of albacore tuna pepsin could recover residual collagen from acid treated skin, thereby increasing total extraction yield. However, pepsin might alter collagen structure, which governed the molecular properties of resultant collagen to some degree.

CHAPTER 8

TYPE I COLLAGEN FROM THE SKIN OF ORNATE THREADFIN BREAM (*NEMIPTERUS HEXODON*): CHARACTERISTICS AND EFFECT OF PEPSIN HYDROLYSIS

8.1 Abstract

Type I collagen from the skin of ornate threadfin bream (*Nemipterus hexodon*) was isolated by acid extraction, followed by purification using salt fractionation and ion-exchange chromatography, respectively. Purified type I collagen contained $[\alpha1(I)]_2\alpha2(I)$ as the dominant components with the co-presence of $\alpha1(I)\alpha2(I)\alpha3(I)$. It was rich in glycine and alanine with high content of imino acids (188 residues/1000 residues). The maximum transition temperature (T_m) and total denaturation enthalpy (ΔH) of purified type I collagen was 33.35 °C and 0.819 J/g, respectively. The conversion factor of hydroxyproline to collagen was 12.72. Based on zeta-potential analysis, the isoelectric point (pI) of purified type I collagen was estimated to be 6.40. After hydrolysis of purified type I collagen using pepsin, the band intensity ratios of α1/α2 chains were increased ($P < 0.05$) with the concomitant decrease in band intensity of higher molecular weight cross-linked components. The cross-linked components were effectively hydrolyzed by pepsin 1 and 2 from skipjack tuna stomach and porcine pepsin at 4 °C without the cleavage of β and α-chains. At 50 °C, purified type I collagen was more susceptible to porcine pepsin hydrolysis, followed by skipjack tuna pepsin 2 and 1, respectively.

8.2 Introduction

Collagen is the fibrous protein that contributes to the unique physiological functions of connective tissues in skins, tendons, bones, cartilages, etc. and is associated with toughness of mammalian muscle (Foegeding *et al.*, 1996; Kittiphattanabawon *et al.*, 2010; Muyonga *et al.*, 2004; Ogawa *et al.*, 2003; Yan *et al.*, 2008). The structural unit of collagen is tropocollagen, a rod-shaped protein consisting of three polypeptides unit (called α chains) intertwined to form a triple-helical structure (Wong, 1989). Each polypeptide chain forms a left-handed helix and consists of repeating triplets, $(Gly\text{-}X\text{-}Y)_n$, where X and Y are, with a high possibility, proline or hydroxyproline (Ogawa *et al.*, 2003). The repetitive occurrence of glycine is absent in the first 14 amino acid residues from N-terminus and the first 10 amino acids from the C-terminus. These end portions are termed "telopeptides" (Foegeding *et al.*, 1996). The covalent cross-links at telopeptide regions between collagen molecules are formed by the condensation of aldehyde groups as well as the formation of a Schiff's base when the aldehyde reacts with an amino group (Foegeding *et al.*, 1996; Jongjareonrak *et al.*, 2005). These cross-linking molecules generally cause the decrease in solubility of collagen (Belitz *et al.*, 2004; Foegeding *et al.*, 1996). At least 19 variants of collagen (types I–XIX) have been reported (Bailey *et al.*, 1998). Type I collagen, $[\alpha1(I)]_2\alpha2$, is the major collagen found predominantly in the connective tissues such as skin, bone, and tendon, and contains two identical α1(I) chains and one α2 chain (Trelstad *et al.*, 1976). Chromatographic and electrophoretic analyses revealed that most of the teleost skin collagens also contained a unique subunit, α3 (Kimura *et al.*, 1987). The existence of $\alpha1\alpha2\alpha3$ and $(\alpha1)_2\alpha2$ heterotrimer of type I collagen from the skin of some teleostean and chondrostean fish species has been reported (Kimura and Ohno, 1987; Kimura *et al.*, 1987; Kimura, 1992).

Currently, the increasing attention of alternative sources for replacement of mammalian collagen has been paid, especially from seafood processing by-products. Fish skin, a by-product from fish processing, is one of alternative sources for collagen preparation. Collagens from several fish species such as arabesque greenling (Nalinanon *et al.*, 2010b), brownbanded bamboo shark

(Kittiphattanabawon *et al.*, 2010), cod (Duan *et al.*, 2009), eagle ray (Bae *et al.*, 2008), walleye pollock (Yan *et al.*, 2008), deep-sea red fish (Wang *et al.*, 2007), bigeye snapper (Benjakul *et al.*, 2010; Kittiphattanabawon *et al.*, 2005; Nalinanon *et al.*, 2007), channel catfish (Liu *et al.*, 2007), Pacific whiting (Kim and Park, 2004), black drum (Ogawa *et al.*, 2003), sheephead seabream (Ogawa *et al.*, 2003), ocellate puffer fish (Nagai *et al.*, 2002) and Japanese seabass, chub mackerel and bullhead shark (Nagai and Suzuki, 2000), have been isolated and characterized. Pepsin, mainly from porcine stomach, was used to improve the extraction efficiency of collagen for a decade. Recently, pepsins from the stomach of several fish species such as bigeye snapper, albacore tuna, tongol tuna and skipjack tuna, have also been used as the aid for collagen extraction (Benjakul *et al.*, 2010; Nalinanon *et al.*, 2007; Nalinanon *et al.*, 2008; Nalinanon *et al.*, 2010b). Nalinanon *et al.* (2008b) found that albacore tuna pepsin exhibited the comparable extraction efficiency of collagen from the skin of threadfin bream to porcine pepsin and no detrimental effect on the integrity of resulting collagen was observed. Nevertheless, pepsin from skipjack tuna caused the degradation of α and β components. The differences in hydrolysis of collagen matrix by pepsin might be dependent on several factors such as source and type of collagen, type of pepsin, hydrolysis condition, etc. Therefore, the objective of this study was to isolate and characterize the major collagen from the skin of ornate threadfin bream (*Nemipterus hexodon*), one of fish species commonly used for surimi production in Thailand, as well as to demonstrate the hydrolysis of type I collagen by different pepsins, including porcine pepsin, skipjack tuna pepsin 1 and 2.

8.3 Materials and Methods

8.3.1 Chemicals

Bovine hemoglobin, *β*-mercaptoethanol (βME), type I collagen from calf skin and pepsin from porcine stomach mucosa (EC 3.4.23.1), (750 units/mg dry matter) were purchased from Sigma Chemical Co. (St. Louis, MO, USA.). Trichloroacetic acid, disodium hydrogen phosphate, sodium citrate, and acetic acid were obtained from Merck (Darmstadt, Germany). Coomassie Blue R-250 and

N,N,N′,N′-tetramethyl ethylene diamine (TEMED) were procured from Bio-Rad Laboratories (Hercules, CA, USA). Sodium dodecyl sulfate (SDS) and bovine serum albumin were obtained from Fluka (Buchs, Switzerland). High-molecular-weight protein markers, DEAE-sephacel and Sephadex G-75 were purchased from GE Healthcare UK Limited (Buckinghamshire, UK). TOYOPEARL® CM-650M was purchased from Tosoh Corporation (Tokyo, Japan).

8.3.2 Preparation of fish stomach and skin

Stomach of skipjack tuna (*Katsuwonus pelamis*) was obtained from Tropical Canning (Thailand) Public Co., Ltd., Songkhla, Thailand. The sample was packed in polyethylene bag, kept in ice with a solid/ice ratio of 1:2 (w/v) and transported to the Department of Food Technology, Prince of Songkla University, Hat Yai, Thailand within 1 h. Upon the arrival, the stomach was cleaned with cold water and placed in polyethylene bag. The stomach sample was stored at -20 °C until use.

Ornate threadfin bream (*Nemipterus hexodon*) was obtained after being off-loaded for 6 h from the dock in Songkhla, Thailand. The fish were kept in ice with a solid/ice ratio of 1:2 (w/v) and transported to the Department of Food Technology, Prince of Songkla University, Hat Yai, Thailand within 30 min. Upon the arrival, the skin was removed and washed with cold water, drained and cut into small pieces (0.5 × 0.5 cm^2) using the scissor. The skin sample was kept in polyethylene bag and stored at -20 °C until use.

8.3.3 Preparation of pepsins

8.3.3.1 Isolation of pepsins

Frozen stomach was thawed using running water (26-28 °C) until the core temperature reached -2 to 0 °C. The sample was cut into pieces with a dimension of 0.5 × 0.5 cm^2. Sample was finely ground in liquid nitrogen using a National Model MX-T2GN blender (Taipei, Taiwan). Sample was then lyophilized using a SCANVAC CoolSafe™ freeze-dryer (CoolSafe 55, ScanLaf A/S, Lynge, Denmark).

To prepare the skipjack pepsin extract, stomach powder was suspended in 20 mM sodium phosphate buffer (pH 7.0) containing 1 mM PMSF at a ratio of 1:19 (w/v) and stirred continuously at 4 °C for 3 h. The suspension was centrifuged for 20 min at 4 °C at 20,000 ×*g* (Avanti® J-E, Beckman Coulter, Inc., Palo Alto, CA, USA) to remove the tissue debris. The supernatant was collected and then adjusted to pH 4 with 1 M HCl. The mixture was allowed to stand at 4 °C for 30 min. The treated stomach extract was then centrifuged at 20,000 ×*g* for 15 min at 4 °C. To fully activate pepsin, the pH of acidified supernatant was adjusted to pH 2 with 1 M HCl and gently stirred for 30 min at 4 °C. The enzyme solution was collected in the same manner as described previously prior to dialysis against 60 volumes of 20 mM sodium phosphate buffer (pH 5.0) referred to as 'starting buffer; SB' for 12 h at 4 °C. The dialysate was collected and referred to as "skipjack pepsin extract".

To prepare the porcine pepsin solution, the enzyme powder (1.0 g) was dissolved with 120 mL of SB, followed by centrifugation at 4 °C for 30 min at 20,000 ×*g*. The supernatant was collected and used for further study.

8.3.3.2 Partial purification of pepsins

All steps of purification were carried out at 4 °C. Skipjack pepsin extract was applied onto DEAE-sephacel column (1.6 × 18 cm), previously equilibrated with SB. After the column was washed with SB until A_{280} was below 0.05, the elution was performed with a linear gradient of 0-0.5 M NaCl in SB at a flow rate of 0.5 mL/min. Fractions of 3 mL were collected and those with proteolytic activity were pooled. Two activity peaks (skipjack tuna pepsin 1 and 2, referred to as SP1 and SP2, respectively) were obtained and the pooled fractions from each peak were dialyzed with 60 volumes of SB for 12 h. The dialysates were then subjected to lyophilization. Lyophilized fractions were dissolved in SB and chromatographed onto Sephadex G-75 (1.6 × 70 cm) column, followed by the elution with SB at the flow rate of 0.5 mL/min. Fractions of 3 mL were collected and the fractions with proteolytic activity were combined and used for further study.

Porcine pepsin solution was purified in the same manner. The purified porcine pepsin fraction was collected and referred to as "PP".

During purification, protein concentration was measured by monitoring the absorbance at 280 nm, and the proteolytic activity was determined using hemoglobin as a substrate at pH 2.0 and 50 °C.

8.3.4 Enzyme assay

Proteolytic activity of pepsin extract was determined using hemoglobin as a substrate according to the method of Nalinanon *et al.* (2010c). To initiate the reaction, 200 μL of enzyme solution were added into the assay mixture containing 200 μL of 2% hemoglobin, 200 μL of distilled water and 625 μL of reaction buffer. Appropriate dilution was made to ensure that the amount of enzyme was not excessive for available substrate in the assay system. The reaction was conducted at pH 2.0 and 50 °C for 20 min. To terminate enzymatic reaction, 200 μL of 50% (w/v) trichloroacetic acid (TCA) were added. Unhydrolyzed protein substrate was allowed to precipitate for 1 h at 4 °C, followed by centrifuging at 4,250 ×*g* for 10 min using a MIKRO 20 centrifuge (Hettich Zentrifugen, Tuttlingen, Germany). The oligopeptide content in the supernatant was measured at 280 nm. One unit of activity was defined as the amount causing an increase of 1.0 in absorbance at 280 nm per min. A blank was run in the same manner, except that the enzyme was added into the reaction mixture after the addition of 50% (w/v) TCA.

8.3.5 Preparation of type I collagen from the skin of ornate threadfin bream

8.3.5.1 Isolation of skin collagen

Acid solubilized collagen from the skin of ornate threadfin bream was isolated following the method of Nalinanon *et al.* (2007) with some modifications. All procedures were performed at 4 °C. To remove non-collagenous proteins, the skin was mixed with 0.1 N NaOH using a sample/alkaline solution ratio of 1:10 (w/v). The mixture was stirred continuously for 6 h. The alkaline solution was changed every 2 h. Then, the alkaline treated skin was washed with cold water until neutral or faintly basic pHs of wash water were obtained. The treated skin was then defatted with 10%

(v/v) 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 3 times prior to lyophilization.

To extract the collagen, the lyophilized skin was soaked in 0.5 M acetic acid with a sample/solution ratio of 1:100 (w/v) for 48 h with gentle stirring. The mixture was then centrifuged at 20,000 ×*g* for 1 h at 4 °C. The supernatant was referred to as 'acid solubilized collagen, ASC'.

8.3.5.2 Fractionation of type I collagen

Type I collagen was fractionated according to the method of Yata *et al.* (2001) with a slight modification. ASC was salted out by adding NaCl to a final concentration of 2.0 M followed by centrifugation at 10,000 ×*g* for 20 min. The precipitate was further extracted with 0.5 M acetic acid containing 11% (w/v) ammonium sulfate for 24 h. The mixture was then centrifuged at 10,000 ×*g* for 20 min. This procedure was repeated 3 times. The resultant precipitate was referred to as "type I collagen".

8.3.5.3 Purification of type I collagen

Fractionated type I collagen was dissolved in 0.5 M acetic acid and dialyzed against 60 volumes of the starting buffer (50 mM sodium acetate, pH 4.8) for 48 h with the change of dialysis solution every 6 h. The dialysate was applied to a TOYOPEARL® CM-650M column (1.8 × 20 cm) previously equilibrated with 10 volumes of the starting buffer at a flow rate of 60 mL/h. After loading, the unbound proteins were washed by the same buffer until A_{230} was less than 0.05. Elution was achieved with a linear gradient of 0–0.5 M NaCl at a flow rate of 60 mL/h with a total volume of 270 mL. The fractions (3 mL) were collected and measured for the absorbance at 230 nm by a spectrophotometer (UV-1800, Shimadzu, Kyoto, Japan). Appropriate fractions were pooled and dialyzed against distilled water, followed by lyophilization. The lyophilized collagen was referred to as "purified type I collagen".

8. 3.6 Characterization of type I collagen

8.3.6.1. Hydroxyproline content and conversion factor for collagen

Purified type I collagen was dehydrated with acetone prior to determination of hydroxyproline content as described by Nalinanon *et al.* (2007). The hydroxyproline content was calculated and expressed as mg/g of dried purified type I collagen. The conversion factor of hydroxyproline content to collagen content was calculated as:

$$\text{Conversion factor} = \frac{100}{\text{Hydroxyproline content (\% dry wt.)}}$$

8.3.6.2 UV absorption measurement

Purified type I collagen was dissolved in 0.5 M acetic acid to obtain a concentration of 1 mg/mL. The solution was placed into a quartz cell with a path length of 1 cm. UV absorption spectrum of collagen was measured using a spectrophotometer. Prior to measurement, the base line was set with 0.5 M acetic acid. The spectrum was obtained by scanning the wavelength in the range of 190–350 nm with a scan speed of 50 nm min^{-1} at room temperature.

8.3.6.3 Amino acid analysis

Purified type I collagen was 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).

8.3.6.4 SDS–polyacrylamide gel electrophoresis (SDS–PAGE)

SDS–PAGE was performed by the method of Laemmli (1970). The samples were mixed with 5% (w/v) SDS and heated at 85 °C for 1 h. The mixtures were then centrifuged at 8,500 ×*g* for 5 min to remove undissolved debris. Solubilized samples were mixed at 1:1 (v/v) ratio with the sample buffer (0.5 M Tris–HCl, pH

6.8, containing 4% (w/v) SDS, 20% (v/v) glycerol) in the presence or absence of 10% β-ME. Samples (15 μg protein) were loaded onto a polyacrylamide gel made of 7.5% running gel and 4% stacking gel and subjected to electrophoresis at a constant current of 15 mA/gel, using a Mini-PROTEAN II unit (Bio-Rad Laboratories Inc., Richmond, CA, USA). After electrophoresis, gels were fixed with a mixture of 50% (v/v) methanol and 10% (v/v) acetic acid for 45 min, followed by staining with 0.05% (w/v) Coomassie Blue R-250 in 15% (v/v) methanol and 5% (v/v) acetic acid for 3 h. Finally, they were destained with the mixture of 30% (v/v) methanol and 10% (v/v) acetic acid for 45 min. High-molecular-weight protein markers (GE Healthcare UK Limited, Buckinghamshire, UK) were used to estimate the molecular weight of proteins. Type I collagen from calf skin was used as a standard. Gels were imaged using a Canon image scanner (CanoScan LiDE25, Canon Inc., Tokyo, Japan) and band intensities were quantified with the public domain digital analysis software, ImageJ (ImageJ 1.42q, National Institutes of Health, Bethesda, MD, USA).

8.3.6.5 Determination of subunit compositions of type I collagen

The analysis of subunit compositions of purified type I collagen was performed according to the method of Kittiphattanabawon *et al.* (2010) and Nalinaon *et al.* (2010a) with a slight modification. Purified type I collagen (20 mg) were dissolved in 10 mL of starting buffer (50 mM sodium acetate buffer, pH 4.8 containing 2 M urea) and incubated at 60 °C for 30 min. The mixtures were centrifuged at 20,000 ×*g* at room temperature (25–26 °C) for 30 min. The supernatant was applied onto a TOYOPEARL® CM-650M column (1.8 × 20 cm) previously equilibrated with 10 volumes of the starting buffer at a flow rate of 60 mL/h. After loading, the unbound proteins were washed by the same buffer until A_{230} was less than 0.05. Elution was achieved with a linear gradient of 0–0.2 M NaCl in the same buffer at a flow rate of 60 mL/h with a total volume of 270 mL. The eluant was monitored at 230 nm and fractions (3 mL) were collected. The selected fractions were subjected to SDS–PAGE using 7.5% separating gel and 4% stacking gel as previously described.

8.3.6.6 Differential scanning calorimetry (DSC)

DSC analysis of collagen samples was carried out following the methods of Nalinanon *et al.* (2007) with a slight modification. The samples were rehydrated by adding 0.05 M acetic acid to dried samples at a solid/solution ratio of 1:40 (w/v). The mixture was allowed to stand for 2 days at 4 °C. DSC analysis was performed using a differential scanning colorimeter (Model DSC 7, Norwalk, CT, USA). Temperature calibration was done using the indium thermogram. The collagen solutions (5–10 mg) were accurately weighed into aluminium pans and sealed. The samples were scanned at 1 °C/min over the range of 20–50 °C using ice water as the cooling medium. An empty pan was used as the reference. The maximum transition temperature (T_m) was estimated from the DSC thermogram. Total denaturation enthalpy (ΔH) was estimated by measuring the area in the DSC thermogram.

8.3.6.7 Measurement of zeta (ζ) potential

Purified type I collagen was dissolved in 0.5 M acetic acid at a concentration of 0.5 mg/mL. The mixture was stirred at 4 °C for 12 h. The ζ-potential of the sample (20 mL) was measured using a Zeta potential analyzer (ZetaPALS, Brookhaven Instruments Co., Holtsville, NY, USA). ζ-potential of samples adjusted to different pHs with 1.0 M Nitric acid or 1.0 M KOH using an Autotitrator (BI-ZTU, Brookhaven Instruments Co., Holtsville, NY, USA) was determined. The isoelectric point (p*I*) was estimated from pH rendering zero ζ-potential.

8.3.7 Hydrolysis of type I collagen by pepsin

PP, SP1 or SP2 (4 units) was added to 2 mL of reaction mixture, containing 8 mg of purified type I collagen in 0.5 M acetic acid, pH 2.0. The hydrolysis was conducted by incubating the mixture at 4 °C for 24 h or at 50 °C for 0, 0.5, 1, 2 and 3 h. The control was performed by incubating the reaction mixture at 4 °C for 24 h or 50 °C for 3 h without the addition of pepsins. At the designated time, 200 μL of reaction mixture was taken and mixed with 100 μL of 1 N NaOH containing 1% (w/v) SDS in order to terminate pepsin activity. The mixture was further mixed with 100 μL of SDS-PAGE sample buffer and boiled for 1 min. The

reaction mixture was analyzed by SDS-PAGE using 7.5% separating gel and 4% stacking gel as previously described.

8.3.8 Statistical analysis

All data were subjected to Analysis of Variance (ANOVA) and the differences between means were evaluated by Duncan's multiple range test. SPSS statistic program (SPSS 11.0 for Windows, SPSS Inc., Chicago, IL, USA) was used for data analysis.

8.4 Results and Discussion

8.4.1 Extraction of ASC and purification of type I collagen

ASC from the skin of ornate threadfin bream was extracted with the yield of 24.9 ± 1.1% (dry wt. the skin). Hydroxyproline content in the skin and ASC was 49.5 ± 0.4 and 72.3 ± 0.5 mg/g dry wt., respectively. The hydroxyproline content of ASC increased by approximately 1.46-fold, compared with that found in the skin. The result suggested that non-collagenous matters could be removed effectively from the skin prior to collagen extraction. To separate type I collagen, ASC was fractionated by NaCl precipitation. The precipitate was further extracted with 0.5 M acetic acid containing 11% (w/v) ammonium sulfate. Yata *et al.* (2001) isolated pepsin solubilized collagen from the skin of common hourse mackerel, yellow sea bream and tiger puffer fish with the same method and found that type I collagen was a major collagen in precipitate, whereas the supernatant contained type V collagen. After salt fractionation, the supernatant was further precipitated by 20% ammonium sulfate. No collagen was recovered, suggesting that very low or no type V collagen in ASC from the skin of ornate threadfin bream was solubilized by acetic acid (data not shown). The collected precipitate was subjected to further purification using TOYOPEARL® CM-650M ion-exchange column chromatography (Figure 31). The peak (fractions 50-62) showed the typical SDS-PAGE pattern of type I collagen with two different α bands, α1(I) and α2(I) (see inset). It also contained β and γ

components as well as other higher molecular weight crosslinked components. The band intensity of α1 was approximately 2-fold higher than that of α2, indicating the existence of $[\alpha1(I)]_2\alpha2(I)$ in collagen triple helix. Fractions no. 50-62 containing collagen components were pooled and used for further study. Based on the precipitation by ammonium sulfate at acidic pH, collagen fraction obtained could be classified as type I collagen. Purified type I collagen had the higher hydroxyproline content (78.6 ± 0.3 mg/g dry wt.) in comparison with that of ASC ($P < 0.05$), indicating a greater purity in the former. When the absorbance spectrum was determined for purified type I collagen, the distinct absorption peak was observed at 230 nm. This was mainly attributed to the absorption of peptide bonds in the polypeptides chains of collagen. No absorption peak was detected at 280 nm (data not shown). Thus, type I collagen from the skin of ornate threadfin bream was obtained with negligible contamination of other proteins.

With reference to hydroxyproline content, the conversion factor of hydroxyproline to collagen from the skin of ornate threadfin bream was calculated to be 12.72. The conversion factor of collagen from other sources such as Baltic cod backbone (15.7) (Żelechowska *et al.*, 2010) and skin (14.7) (Sadowska *et al.*, 2003) has been reported. This factor might be useful for estimation of the collagen content in raw material and the yield of extraction as well as purity assessment of resultant collagen.

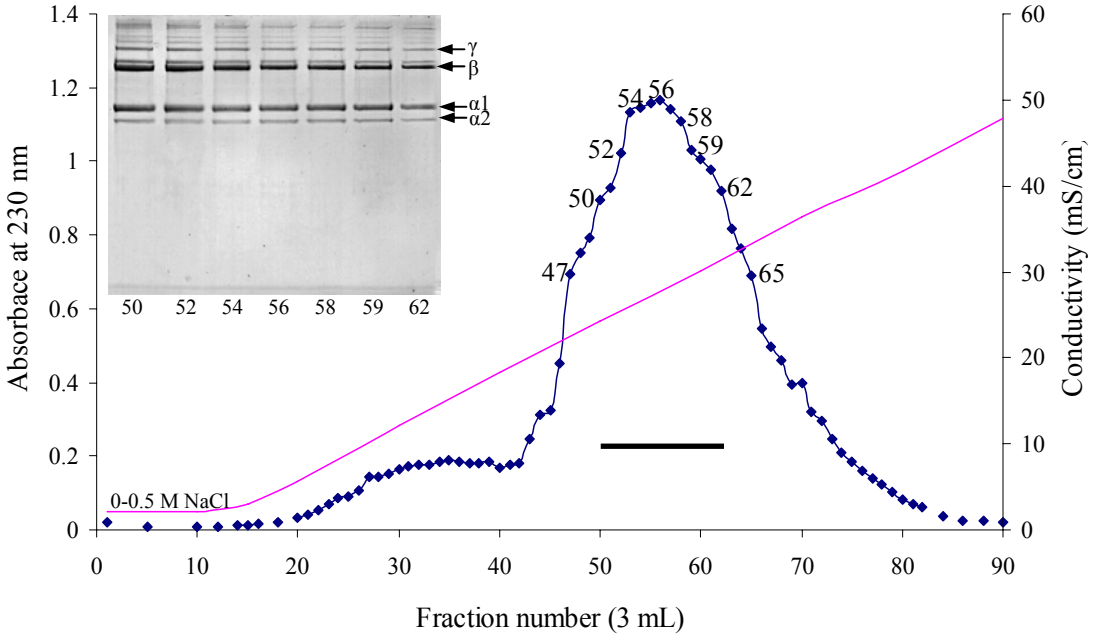

Figure 31. TOYOPEARL® CM-650M ion-exchange column chromatography of type I collagen isolated from the skin of ornate threadfin bream. The column (1.8 × 20 cm) was equilibrated with 50 mM sodium acetate, pH 4.8. Adsorbed proteins were eluted with a linear gradient of 0–0.5 M NaCl over a total volume of 270 mL at a flow rate of 60 mL/h. The inset shows the SDS–PAGE pattern (7.5% separating gel and 4% stacking gel) of the fractions indicated by the numbers.

8.4.2 Subunit composition of purified type I collagen

The elution profiles of purified type I collagen on the TOYOPEARL® CM-650M column after being denatured by heat treatment in the presence of 2 M urea are shown in Figure 32. Three peaks containing different collagen components were obtained. The chromatographic fractions indicated by numbers were subjected to SDS-PAGE analysis as shown in the inset. The first peaks (fraction no. 37) contained only a band of α chain. The second peak (fraction no. 41) consisted of two distinct components, β11 (dimer of α1) and α chains. High MW component, γ-chain and β12 (dimer of α1 and α2) were concentrated in fraction no. 47-51 of the third peak, while α2 was observed in fraction no. 49-51. Since the α chains found in fraction no. 37 and

41 were eluted by different NaCl concentrations but they had similar molecular mobility, both α1 and α3 chains were most likely present in purified collagen. Kimura *et al.* (1987) and Matsui *et al.* (1991) reported that the electrophoretic mobility in SDS-PAGE of α3 from chum salmon, as well as other teleosts, was almost identical to that of α1. Therefore, purified collagen from the skin of ornate threadfin bream not only comprised $[\alpha1(I)]_2\alpha2(I)$ but also $\alpha1(I)\alpha2(I)\alpha3(I)$. Existence of two molecular forms, $[\alpha1(I)]_2\alpha2(I)$ and $\alpha1(I)\alpha2(I)\alpha3(I)$, were reported in fish type I collagen (Kimura *et al.*, 1987). The α3 chain was reported as the intermediate in chemical nature between α1 and α2 (Matsui *et al.*, 1991).

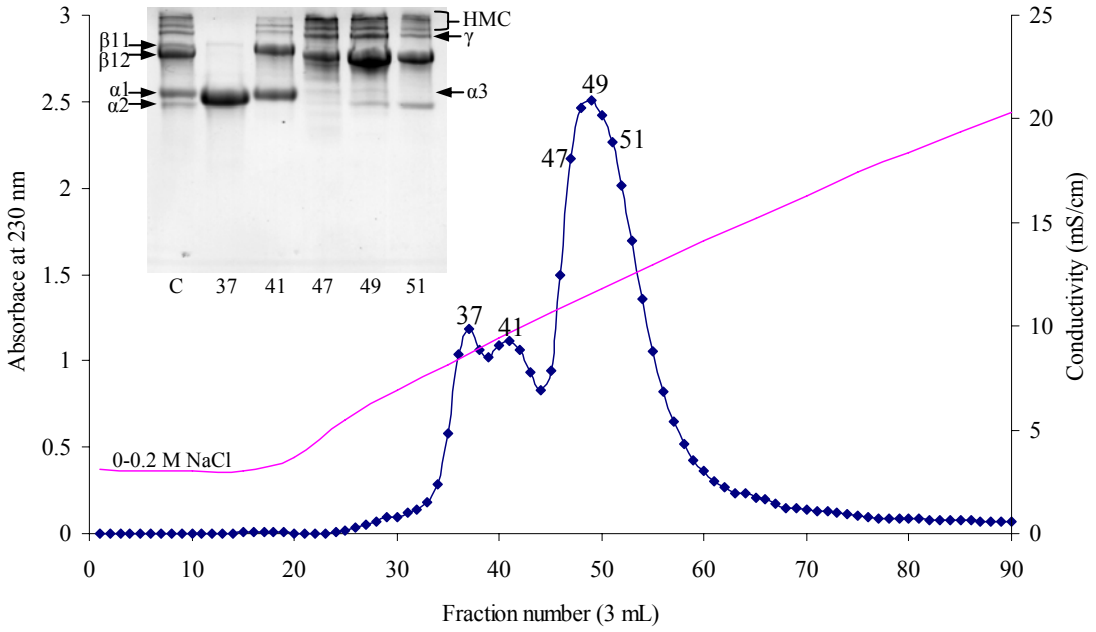

Figure 32. TOYOPEARL® CM-650M ion-exchange column chromatography of denatured type I collagen from the skin of ornate threadfin bream. The column (1.8 × 20 cm) was equilibrated with 50 mM sodium acetate, pH 4.8 containing 2 M urea. Adsorbed proteins were eluted with a linear gradient of 0–0.2 M NaCl over a total volume of 270 mL at a flow rate of 60 mL/h. The inset shows the SDS–PAGE pattern (7.5% separating gel and 4% stacking gel) of the fractions indicated by the numbers. C and HMC denote collagen and high MW cross-linked components, respectively.

8.4.3 Amino acid composition of purified type I collagen

The amino acid composition of purified type I collagen from the skin of ornate threadfin bream expressed as residues per 1000 total amino acid residues is shown in Table 19. The purified type I collagen was rich in glycine, alanine and proline. It had relatively low contents of tyrosine and histidine and it contained no cysteine and tryptophan. The results were in accordance with those of type I collagens from the skin of other fish species such as common horse mackerel, yellow sea bream, tiger puffer fish (Yata *et al.*, 2001), black drum, sheephead seabream (Ogawa *et al.*, 2003), brownstripe red snapper (Jongjareonrak *et al.*, 2005) and arabesque greenling (Nalinanon *et al.*, 2010b). The imino acid (proline and hydroxyproline) content of purified type I collagen was 188 residues/1000 residues, which was slightly lower than that of type I collagen from calf skin (215 residues/1000 residues) (Ogawa *et al.*, 2003), young and adult Nile perch (193-200 residues/1000 residues) (Muyonga *et al.*, 2004) and bigeye snapper (193 residues/1000 residues) (Kittiphattanabawon *et al.*, 2005). However, imino acid content of purified collagen was relatively higher than those reported for collagen from the skin of cold water fish species such as arabesque greenling (Nalinanon *et al.*, 2010b), cod (Duan *et al.*, 2009), deep-sea red fish (Wang *et al.*, 2007) and Pacific whiting (Kim and Park, 2004), which had imino acid content ranging from 154 to 165 residues/1000 residues. Imino acids contribute to the stability of helical structure of collagen (Ikoma *et al.*, 2003; Jongjareonrak *et al.*, 2005). The stability of collagen was proportional to the total content of imino acids (Kittiphattanabawon *et al.*, 2010). Pyrrolidine rings of proline and hydroxyproline impose restrictions on the conformation of the polypeptide chain and help to strengthen the triple helix (Bae *et al.*, 2008; Wong, 1989). The difference in imino acid content among animals was associated with the different living environments (Foegeding *et al.*, 1996; Kittiphattanabawon *et al.*, 2010; Nalinanon *et al.*, 2010b; Regenstein and Zhou, 2007). Hydroxylysine (8 residues/1000 residues) was found in purified type I collagen, suggesting the partial cross-linking of collagen via covalent bond (Mechanic *et al.*, 1987; Wong, 1989).

Table 19. Amino acid composition of purified type I collagen from the skin of ornate threadfin bream (residues/1000 residues)

Amino acid	Purified type I collagen
Alanine	134
Arginine	53
Aspartic acid/Asparagine	49
Cysteine	0
Glutamic acid/Glutamine	72
Glycine	321
Histidine	5
Isoleucine	9
Leucine	23
Lysine	27
Hydroxylysine	8
Methionine	14
Phenylalanine	14
Hydroxyproline	84
Proline	104
Serine	35
Threonine	22
Tyrosine	4
Tryptophan	0
Valine	21
Total	**1000**
Imino acids[a]	188

[a] Imino acids include proline and hydroxyproline

8.4.4 Thermal stability of purified type I collagen

DSC thermograms of purified type I collagen from the skin of ornate threadfin bream and type I collagen from calf skin rehydrated in 0.05 M acetic acid are shown in Figure 33. The maximum transition temperature (T_m) and total denaturation enthalpy (ΔH) of purified type I collagen was 33.35 °C and 0.819 J/g, respectively, which were lower than that of type I collagen from calf skin (T_m = 39.27 °C; ΔH = 1.204 J/g). This might be owing to the lower content of imino acids (hydroxyproline and proline) of collagen from the skin of ornate threadfin bream. Thermal stability of collagen is associated with the restriction of the secondary structure of the polypeptide chain governed by the pyrrolidine rings of proline and hydroxyproline and partially by the hydrogen bonding through the hydroxyl group of

hydroxyproline (Benjakul *et al.*, 2010; Piez and Gross, 1960). T_m of purified type I collagens from the skin of ornate threadfin bream was similar to that of collagen from subtropical and tropical species such as bigeye snapper (32.5 °C) (Nalinanon *et al.*, 2007) and channel catfish (32.5 °C) (Liu *et al.*, 2007), black drum (34.2 °C) (Ogawa *et al.*, 2003), sheephead seabream (34 °C) (Ogawa *et al.*, 2003), eagle ray (34.1 °C) (Bae *et al.*, 2008) and brownbanded bamboo shark (Kittiphattanabawon *et al.*, 2010). Lower denaturation temperatures of skin collagen from several cold and temperate water fishes including cod (15 °C) (Duan *et al.*, 2009), arabesque greenling (Nalinanon *et al.*, 2010b), deep-sea redfish (16.1 °C) (Wang *et al.*, 2007), Pacific whiting (21.7 °C) (Kim and Park, 2004), walleye pollock (24.6 °C) (Yan *et al.*, 2008), Japanese seabass (26.5 °C), chub mackerel (25.6 °C), bullhead shark (25 °C) (Nagai and Suzuki, 2000) and ocellate puffer fish (28 °C) (Nagai *et al.*, 2002), have been reported. The difference in denaturation temperature among collagens from different fish species was correlated with the imino acid content, body temperature and environmental temperature (Kittiphattanabawon *et al.*, 2010; Nagai *et al.*, 2002; Rigby, 1968).

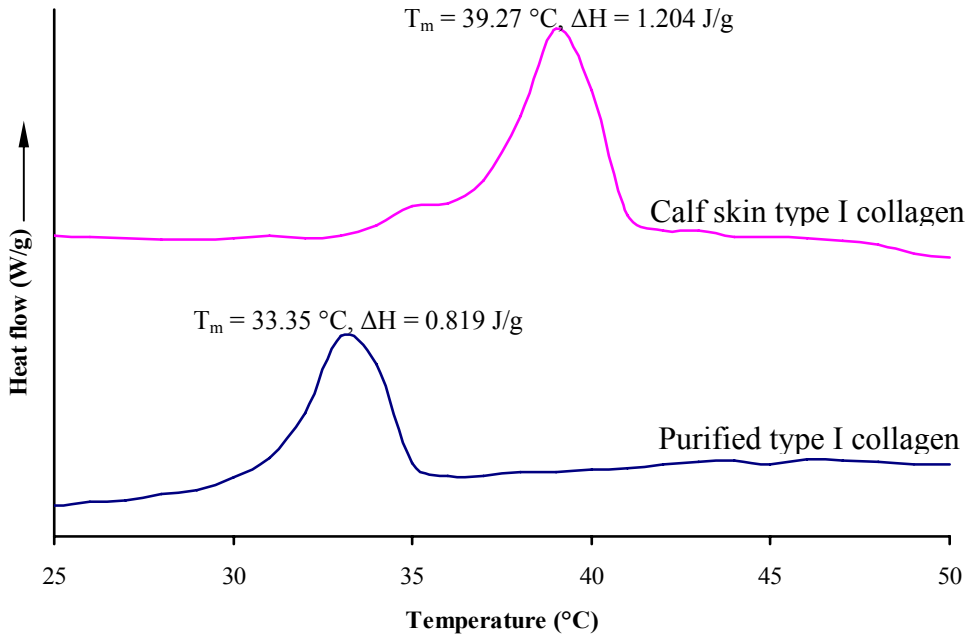

Figure 33. DSC thermograms of purified type I collagen from the skin of ornate threadfin bream and calf skin type I collagen. Collagen samples were rehydrated in 0.05 M acetic acid prior to analysis.

8.4.5 ζ-potential of purified type I collagen

ζ-potential representing the surface charge of purified type I collagen from the skin of ornate threadfin bream at different pHs is shown in Figure 34. The surface net charge of purified type I collagen decreased as the pH increased and it became zero at pH 6.40. The pH, where the ζ-potential is zero, corresponds to pI of the protein (Ma *et al.*, 2009; Nalinanon *et al.*, 2010b). Thus, the pI of purified type I collagen was estimated to be 6.40. Purified type I collagen had a net positive or negative charge when pH values were below and above their pI, respectively. The pI of purified type I collagen was found in a slight acidic pH, which was in accordance with those found in collagen from the skin of arabesque greenling (Nalinanon *et al.*, 2010b) and brownbanded bamboo shark (Kittiphattanabawon *et al.*, 2010). This might be due to higher content of acidic amino acids, aspartic acid and glutamic acid, compared to basic amino acids, including histidine, lysine and arginine (Nalinanon *et al.*, 2010b).

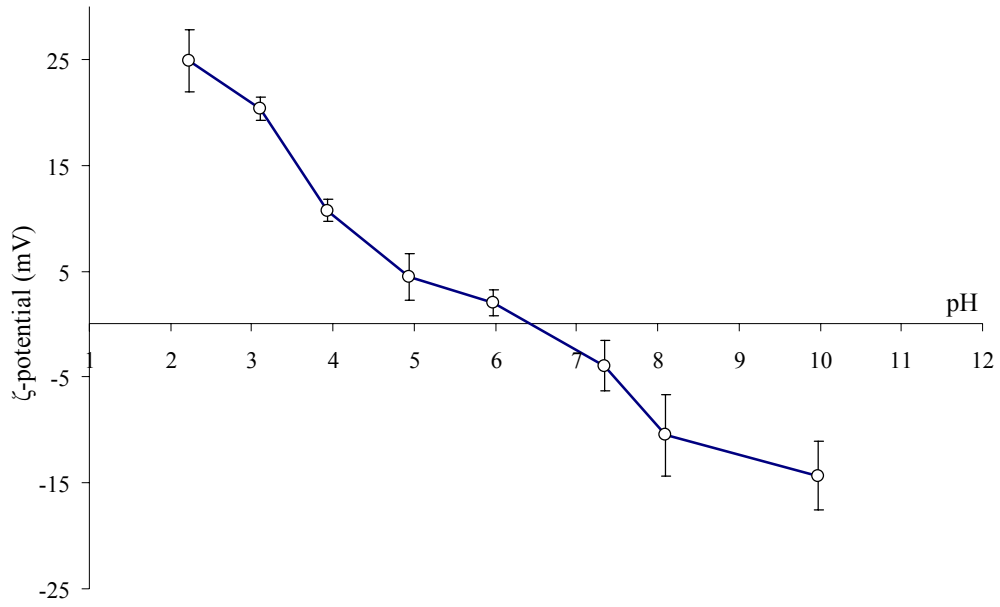

Figure 34. Zeta (ζ) potential of purified type I collagen from the skin of ornate threadfin bream at different pHs. Bars represent the standard deviation (n = 3).

8.4.6 Hydrolysis of purified type I collagen by different pepsins

The degradation patterns of purified type I collagen from the skin of ornate threadfin bream (C-OTB) by porcine pepsin (C-PP), skipjack pepsin 1 (C-SP1) and 2 (C-SP2) at a level of 0.5 unit/mg collagen, for 24 h at 4 °C are shown in Figure 35. This condition was carried out to simulate the hydrolysis of type I collagen for the extraction of pepsin solubilized collagen. The result revealed that the major components including β, α1, and α2 were fully retained (Figure 35a). However, some degradation bands were observed in C-PP, C-SP1 and C-SP2 as evidenced by the increase in band intensity at the dye front. The degradation was more pronounced in C-SP2, in which degradation products with molecular weight (MW) of 67 and 71 kDa were observed. The small peaks of those bands were noticeable in densitogram (Figure 35a and 35b). Pepsin treatment of C-OTB had the impact on the proportion of collagen components. In general, β- and γ-chains (dimer and trimer, respectively) as well as very high (VMC) and high MW components (HMC) in collagens decreased after treatment with all pepsins. Cleavage of telopeptide region more likely resulted in the conversion of cross-links, such as β- and γ-chains, to the α-chain (Figure 35b). C-OTB contained the higher propotion of cross-linked chains (β, γ and VMC) than that of pepsin treated collagen. VMC in C-OTB disappeared when treated with pepsin, regardless of type of pepsin used. This VMC was degraded to smaller components, such as β, α1 or α2, by pepsin. Substantial decreases in VMC, HMC, β and γ chains with the concomitant formation of α1 were observed. The band intensity ratios of α1/α2 chains were significantly increased from 2.18 (C-OTB) to 2.37, 3.00 and 3.45 for C-PP, C-SP1 and C-SP2, respectively (Table 20). The result showed that among pepsin used, SP2 had the greatest ability to cleave intermolecular cross-links in C-OTB to produce a monomeric chain. Among pepsin used, SP1 exhibited less detrimental effect on collagen structure, as evidenced by the higher ratio of collagen components (β, α1 and α2) to that of lower MW components produced from pepsin hydrolysis. The conversion of cross-linked components to monomer chains, by removing the cross-link containing telopeptide, was reported for pepsin solubilized collagens from the skin of deep-sea redfish (Wang *et al.*, 2007), black drum and sheepshead seabream (Ogawa *et al.*, 2003). There were no differences in the relative

mobilities of α1 and α2 chains between C-OTB and C-PP. Slight differences in relative mobilities between C-OTB and collagen with skipjack pepsin treatments (C-SP1 and C-SP2) were observed. The MWs of α1 and α2 chains estimated to be 128 and 116 kDa, respectively, were observed in both C-OTB and C-PP. For C-SP1 and C-SP2, MWs of the α1 chains were estimated to be 126 and 122 kDa, respectively, whereas those of α2 chains were 115 and 112 kDa, respectively. Short peptides with MWs of 2-6 kDa and 1-4 kDa from the telopeptides of α1 and α2, respectively, were produced by the action of pepsin. These results suggested that both pepsins from skipjack tuna partially hydrolyzed C-OTB at either telopeptide regions or the end of helical region. A slight decrease in MW by about 1-2 kDa has been reported for pepsin solubilized collagens from the skin of bigeye snapper, in comparison with that of acid solubilized collagen (Nalinanon *et al.*, 2007). Although no changes in relative mobilities of α-chains in C-PP, a partial cleavage of collagen at telopeptide regions also took place when porcine pepsin was used as evidenced by the increase in α1/α2 proportion. Therefore, different pepsins were able to cleave collagen crosslinks at different positions, particularly at telopeptide region.

Table 20. Band intensity ratios of α1 chain to α2 chain and cross-linked chains to total monomer chains of purified type I collagen from the skin of ornate threadfin bream subjected to hydrolysis by different pepsins.

Components	Band intensity ratio[†]			
	C-OTB	C-PP	C-SP1	C-SP2
α1/α2	2.18 ± 0.10a[‡]	2.37 ± 0.05b	3.00 ± 0.12c	3.45 ± 0.09d
β/(α1+α2)	1.39 ± 0.06c	1.18 ± 0.04b	1.21 ± 0.05b	0.97 ± 0.04a
γ/(α1+α2)	0.33 ± 0.01c	0.18 ± 0.01b	0.16 ± 0.02b	0.11 ± 0.04a
HMC/(α1+α2)	0.62 ± 0.04b	0.59 ± 0.03ab	0.55 ± 0.02a	0.52 ± 0.03a
VMC/(α1+α2)	0.61 ± 0.01b	0.02 ± 0.01a	0.04 ± 0.02a	0.03 ± 0.02a

[†] Mean ± standard deviation (n = 3).

[‡] Different letters in the same row indicate the significant differences ($P < 0.05$).

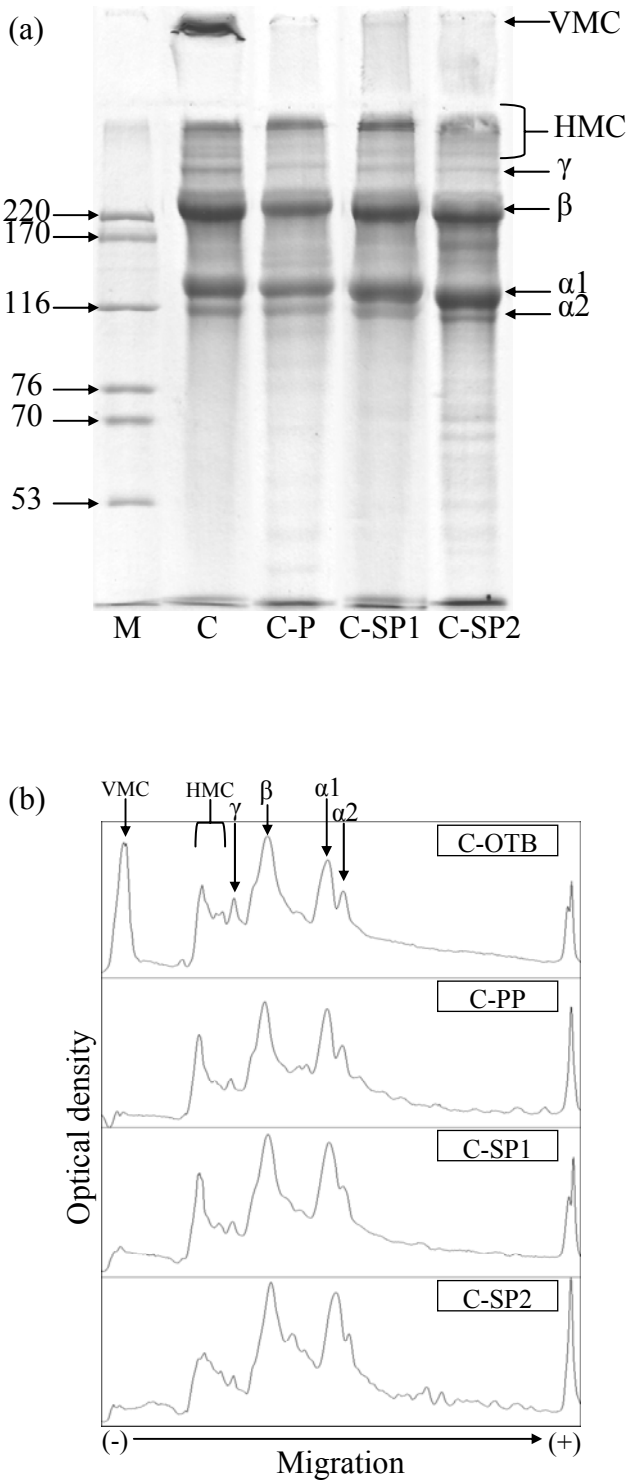

Figure 35. Protein patterns (a) and densitograms (b) of purified type I collagen (C-OTB) and purified type I collagen treated with porcine pepsin (C-PP), skipjack pepsin 1 (C-SP1) and skipjack pepsin 2 (C-SP2) at a level of 0.5 unit/mg collagen for 24 h at 4 °C. VMC and HMC denote very high and high MW cross-linked components of collagen, respectively. M denotes standard protein marker.

Degradation patterns of purified type I collagen from the skin of ornate threadfin bream treated with PP, SP1 and SP2 at a level of 0.5 unit/mg collagen at 50 °C for different times are shown in Figure 36. Hydrolysis was more pronounced with increasing digestion time. Almost HMC and γ-chain disappeared after 30 min of pepsin digestion. The degradation was much higher in purified type I collagen treated with porcine pepsin (Figure 36a), compared with that found in collagen treated with skipjack tuna pepsin. The major components including γ-, β- and α-chains were drastically hydrolyzed by porcine pepsin after 30 min with the concomitant formation of degradation bands with MWs of 47, 52 and 70-101 kDa. Those major components were completely hydrolyzed after 2 h of digestion. Furthermore, α2-chain was more susceptible to porcine pepsin hydrolysis than α1-chain. Purified type I collagen was therefore more resistant to skipjack tuna pepsin hydrolysis (Figure 36b and 36c). The major components were retained after 3 h of skipjack tuna pepsin hydrolysis. The results revealed that SP2 had a slightly greater hydrolysis activity on purified type I collagen than did SP1. The lower band intensity of HMC, β- and α-chain of purified type I collagen treated with SP2 was observed, compared with that of SP1 hydrolysis. The similar result was reported by Nalinanon *et al.* (2010c) who found that purified pepsin 2 from the stomach of skipjack tuna exhibited slightly higher effectiveness for hydrolysis of ASC from the skin of arabesque greenling, compared with purified pepsin 1. Therefore, fish pepsin, especially from skipjack tuna, could be used to increase the extraction efficacy of collagen with the negligible cleavage of the major components of collagen. This revealed the greater advantage of fish pepsin as the aid for the extraction of pepsin solubilized collagen from fish skin.

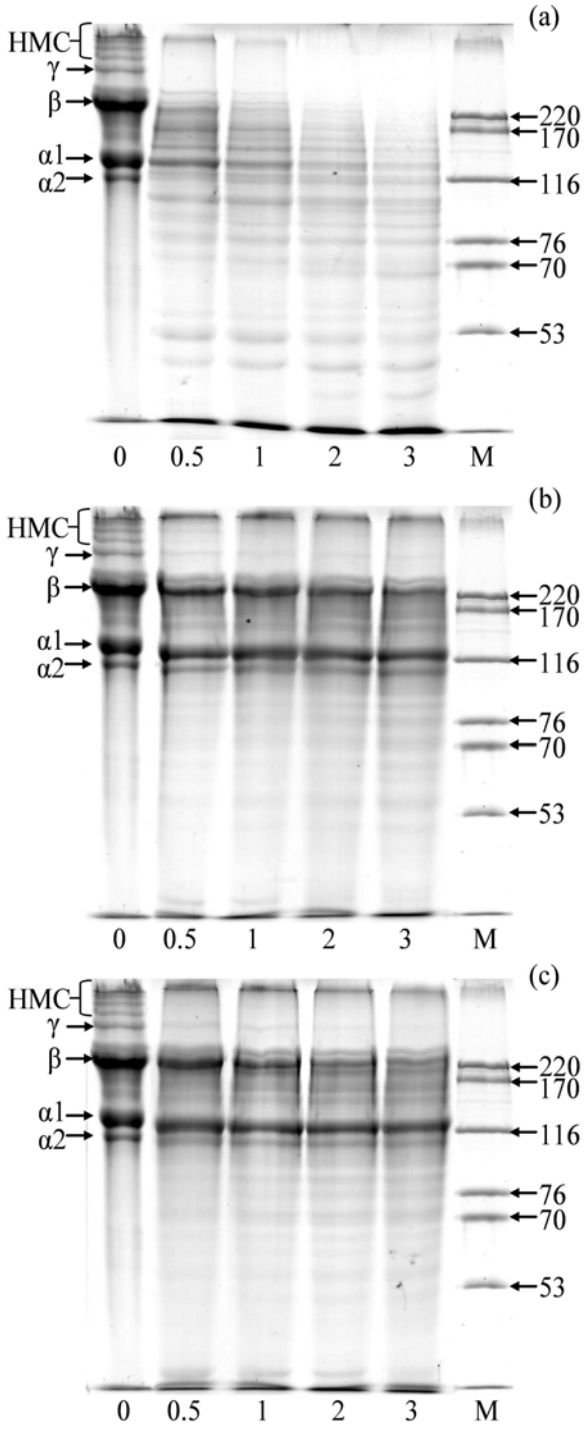

Figure 36. Degradation patterns of purified type I collagen treated with PP (a), SP1 (b) and SP2 (c) at a level of 0.5 unit/mg collagen at 50 °C for different times. Number of each lane denotes pepsin hydrolysis time (h). HMC and M denote high MW cross-linked components of collagen and standard protein marker, respectively.

8.5 Conclusion

ASC from the skin of ornate threadfin bream contained type I collagen with $[\alpha1(I)]_2\alpha2(I)$ as the major components and $\alpha1(I)\alpha2(I)\alpha3(I)$ was also found as minor component. Type I collagen showed the varying susceptibility to hydrolysis by pepsin, depending on hydrolysis temperature and type of pepsin used. Pepsin from the stomach of skipjack tuna showed the greater ability to cleave the cross-linked components without the cleavage of major components of collagen, whereas porcine pepsin more likely hydrolyzed all components, especially at high temperature.

CHAPTER 9

FUNCTIONALITIES AND ANTIOXIDATIVE PROPERTIES OF PROTEIN HYDROLYSATES FROM THE MUSCLE OF ORNATE THREADFIN BREAM TREATED WITH PEPSIN FROM SKIPJACK TUNA

9.1 Abstract

Functional properties and antioxidative activities of protein hydrolysates prepared from ornate threadfin bream (*Nemipterus hexodon*) muscle, using skipjack tuna pepsin, with different degree of hydrolysis (DH: 10, 20 and 30%), were determined. The solubility of hydrolysates varied from 71 to 99% in the pH range of 3-9, depending on their DH. Emulsifying and foaming properties of hydrolysates were governed by DH and concentrations used. Hydrolysates with 20% DH had the highest 2,2-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt (ABTS) and 2,2-diphenyl-1-picryl hydrazyl (DPPH) radical scavenging activities. However, chelating activity on Fe^{2+} of hydrolysate increased as DH increased ($P < 0.05$). Size exclusion chromatography of hydrolysate with 20% DH using Sephadex G-25 revealed that antioxidative peptides with molecular weight of approximately 1.3 kDa exhibited the highest ABTS radical scavenging activity. The fraction containing antioxidative peptides was quite stable over a wide pH range (1-10) and had high stability when heated at 100 °C for up to 3 h. *In vitro* simulated gastrointestinal digestion indicated that ABTS radical scavenging activity of the antioxidative peptides was not affected by pepsin hydrolysis, while further digestion by pancreatin enhanced the activity. Therefore, protein hydrolysate from the muscle of ornate threadfin bream produced by skipjack tuna pepsin can be used as a promising source of functional antioxidative peptides.

9.2 Introduction

The uses of enzyme technologies for protein recovery and modification have led to the production of a broad spectrum of food ingredients and industrial products (Kristinsson and Rasco, 2000). Proteases from different sources are commonly applied to obtain a more selective hydrolysis due to their specificity for peptide bonds adjacent to certain amino acid residues (Khantaphant and Benjakul, 2008). Enzymatic hydrolysis of food proteins is an efficient way to recover potent bioactive peptides (Thiansilakul *et al.*, 2007a). Fish protein hydrolysates have been shown to have potential for nutritional or pharmaceutical applications (Thiansilakul *et al.*, 2007a; Wu *et al.*, 2003). Numerous peptides derived from hydrolyzed food proteins have been shown to have antioxidative activities. Fish protein hydrolysates such as skin gelatin hydrolysate from brownstripe red snapper (Khantaphant and Benjakul, 2008) or meat protein hydrolysates from yellow travelly (Klompong *et al.*, 2007; Klompong *et al.*, 2009), round scad (Thiansilakul *et al.*, 2007a; Thiansilakul *et al.*, 2007b), mackerel (Wu *et al.*, 2003) and loach (You *et al.*, 2010) have been reported to exhibit antioxidative activity. Fish protein hydrolysates can be applied in food systems, comparable to pertinent food protein hydrolysates (Kristinsson and Rasco, 2000).

Thailand is the world's largest producer and exporter of canned tuna. The most commercially important tuna species for the Thai tuna industry is skipjack tuna, followed by yellow-fin tuna and tongol tuna, accounting for over 80% of Thai frozen tuna imports (Nalinanon *et al.*, 2010a). Due to a large amount of tuna viscera discarded during manufacturing, it can serve as a promising source of pepsin for further applications. Recovery of pepsin from tuna stomach is an approach to minimize the economic and ecological problems of this processing waste. Additionally, fish protein hydrolysate with bioactivity prepared with the aid of fish proteases can be obtained as the new value-added product with high market value (Khantaphant and Benjakul, 2008). Skipjack tuna pepsins were recently purified and characterized by Nalinanon *et al.* (2010c). Pepsins from skipjack tuna effectively hydrolyzed several protein substrates including bovine serum albumin, egg white,

natural actomyosin from brownstripe red snapper muscle and acid-solubilized collagen from arabesque greenling skin (Nalinanon *et al.*, 2010b).

Ornate threadfin bream (*Nemipterus hexodon*) belongs to the order Perciformes and is a member of the Nemipteridae family, found in tropical and sub-tropical Indo-West Pacific. Ornate threadfin bream is the potential raw material and widely used for surimi production in Thailand. However, the price of surimi has been fluctuated, depending on the world market. To increase the value of meat from this fish species, the production of protein hydrolysates with nutritive value as well as good functional properties can be an alternative way for its full utilization. The use of fish pepsin recovered from stomach of tuna, a byproduct from canned tuna processing, for hydrolysate production, could lower the cost of commercial proteases. Furthermore, the production of fish protein hydrolysate using tuna pepsin has also not been reported. Therefore, the objectives of this investigation were to study the functionalities and antioxidative properties of the protein hydrolysate prepared from ornate threadfin bream (*N. hexodon*) muscle using skipjack tuna pepsin and to characterize the antioxidative peptides in the resulting hydrolysate.

9.3 Materials and Methods

9.3.1 Chemicals

Bovine hemoglobin, bovine serum albumin (BSA), pepsin from porcine gastric mucosa (EC 3.4.23.1) (P7000, 422 units/mg solid), L-leucine, 2,4,6-trinitrobenzenesulphonic acid (TNBS), 2,2-diphenyl-1-picryl hydrazyl (DPPH), 2,2-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt (ABTS), 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (Trolox) and 3-(2-pyridyl)-5-6-diphenyl-1,2,4-triazine-4′,4″-disulphonic acid sodium salt (ferrozine) were purchased from Sigma Chemical Co. (St. Louis, MO, USA). Pancreatin was obtained from Wako Pure Chemical Industries, Ltd. (Tokyo, Japan). Folin-Ciocalteu's phenol reagent, trichloroacetic acid (TCA), potassium persulfate, sodium sulfite and ferrous chloride were obtained from Merck (Darmstadt, Germany). Sephadex G-25 was purchased from GE Healthcare UK Limited (Buckinghamshire, UK). Sodium dodecyl

sulfate (SDS), Coomassie Blue R-250, and *N,N,N′,N′*-tetramethyl ethylene diamine (TEMED) were procured from Bio-Rad Laboratories (Hercules, CA, USA). All chemicals were of analytical grade.

9.3.2 Preparation of skipjack tuna stomach

Internal organs of skipjack tuna (*Katsuwonus pelamis*) were obtained from Tropical Canning (Thailand) Public Co., Ltd., Songkhla, Thailand. The samples were packed in polyethylene bag, kept in ice with a solid/ice ratio of 1:2 (w/v) and transported to the Department of Food Technology, Prince of Songkla University, Hat Yai within 30 min. Upon arrival, internal organs were excised and the stomach was collected and cut into pieces with a dimension of 0.5 × 0.5 cm^2. Sample was frozen in liquid nitrogen and finely ground using a National Model MX-T2GN blender (Taipei, Taiwan). Sample was then lyophilized using a SCANVAC CoolSafe™ freeze-dryer (CoolSafe 55, ScanLaf A/S, Lynge, Denmark). The dry powder referred to as 'stomach powder' was stored at -20 °C until use.

Ornate threadfin bream (*N. hexodon*) was purchased from the dock in Songkhla, Thailand. The fish, off-loaded approximately 18-24 h after capture, were placed in ice at a fish/ice ratio of 1:2 (w/w) and transported to the Department of Food Technology, Prince of Songkla University, Hat Yai, within 1 h. Upon the arrival, fish were filleted and the ordinary muscle was collected. Size reduction of muscle was carried out by chopping manually into small pieces. The prepared sample was kept in ice until use.

9.3.3 Preparation of pepsin from skipjack tuna stomach and activity assay

To prepare the skipjack tuna pepsin extract, the stomach powder was suspended in 20 mM sodium phosphate buffer (pH 7.0) containing 1 mM PMSF at a ratio of 1:19 (w/v) and stirred continuously at 4 °C for 3 h. The suspension was centrifuged for 20 min at 4 °C at 20,000 ×*g* (Avanti® J-E, Beckman Coulter, Inc., Palo Alto, CA, USA) to remove the tissue debris. The supernatant was collected and then adjusted to pH 4 with 1 M HCl. The mixture was allowed to stand at 4 °C for 30 min.

The prepared extract was then centrifuged at 20,000 ×*g* for 15 min at 4 °C. To fully activate pepsin, the pH of acidified supernatant was adjusted to pH 2 with 1 M HCl and gently stirred for 30 min at 4 °C. The enzyme solution was subjected to centrifugation and the supernatant was collected in the same manner as described previously and referred to as "skipjack tuna pepsin".

Proteolytic activity of skipjack tuna pepsin was determined using hemoglobin as a substrate as described by Nalinanon *et al.* (2010c). The reaction was conducted at pH 2.0 and 50 °C for 20 min. One unit of activity was defined as the amount causing an increase of 1.0 in absorbance at 280 nm per min.

9.3.4 Preparation of protein substrate from ornate threadfin bream muscle

Protein substrate from ornate threadfin bream muscle was prepared by acid solubilization process according to the method of Pérez-Mateos and Lanier (2006) with some modifications. Chopped fish muscle was homogenized with eight volumes of deionized water (0–6 °C) for 2 min in a blender at a maximum speed (type AY46, Moulinex, Groupe SEB Thailand Ltd., Bangkok, Thailand). To solubilize proteins, the pH of the mixture was adjusted to 2.0 using 1 N HCl. The samples were centrifuged at 5,000 ×*g* for 10 min at 4 °C. The middle layer, containing solubilized proteins, was filtered through four layers of cheesecloth. The filtrate was then adjusted to pH 5.5 using 1 N NaOH with gradual stirring prior to centrifugation at 5,000 ×*g* for 10 min at 4 °C. The pellet was dispersed in four volumes of deionized water (0–6 °C) and then the pH of protein suspension was adjusted to 2.0. Acid solubilized protein fraction was used as a protein substrate for preparation of hydrolysate.

9.3.5 Production of protein hydrolysate from ornate threadfin bream muscle

Acid solubilized protein fraction from the muscle of ornate threadfin bream was mixed with McIlvaine buffer (0.2 M disodium hydrogen phosphate and 0.1 M sodium citrate) (pH 2.0) at a ratio of 1:1 (w/v). The mixture was pre-incubated at 50 °C for 15 min. The enzymatic hydrolysis was started by adding skipjack tuna pepsin with the amount (140–650 μL) required to gain 10, 20 and 30% DH into 250

mL of mixture as described by Benjakul and Morrissey (1997). After 1 h of hydrolysis, the enzyme was inactivated by adjusting the pH of the reaction mixture to 7.0 prior to heating at 90 °C for 15 min. The mixture was then centrifuged at 2,000 ×*g* for 10 min. The supernatant was then collected and lyophilized using a freeze-dryer. Lyophilized protein hydrolysate obtained was subjected to analyses.

9.3.6 Determination of α-amino acid content and DH

The α-amino acid content was determined according to the method of Benjakul and Morrissey (1997). To properly diluted hydrolysate samples (125 μL), 2.0 mL of 0.2 M phosphate buffer (pH 8.2) and 1.0 mL of 0.01% TNBS solution were added. The solution was mixed thoroughly and placed in a temperature controlled water bath (Model W350, Memmert, Schwabach, Germany) at 50 °C for 30 min in the dark. The reaction was terminated by adding 2.0 mL of 0.1 M sodium sulfite. The mixtures were cooled at room temperature for 15 min. The absorbance was read at 420 nm and α-amino acid 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 the original acid solubilized protein substrate. L_{max} is total α-amino acid in the original acid solubilized protein substrate obtained after acid hydrolysis (6 N HCl at 100 °C for 24 h).

9.3.7 Functional properties of protein hydrolysate

9.3.7.1 Solubility

To determine protein solubility, hydrolysate samples (10 mg) were dispersed in 8 mL of deionized water and pH of the mixture was adjusted to 3, 5, 7 and 9 with either 1 N HCl or 1 N NaOH. The mixture was stirred at room temperature

for 30 min. The volume of solutions was made up to 10 mL by distilled water, previously adjusted to the same pH as the sample solution prior to centrifugation at 5000 ×*g* for 15 min. Protein content in the supernatant was determined using the Lowry method (Lowry *et al.*, 1951), using bovine serum albumin as a standard. Total protein content in the sample was determined after solubilization of the sample in 0.5 N NaOH. Protein solubility was calculated as follows:

$$\text{Solubility (\%)} = \frac{\text{protein content in supernatant}}{\text{total protein content in sample}} \times 100$$

9.3.7.2 Emulsifying properties

The emulsifying activity index (EAI) and the emulsion stability index (ESI) were determined according to the method of Pearce and Kinsella (1978) with a slight modification. Soybean oil (2 mL) and protein hydrolysate solutions (0.10, 0.25 and 0.50% protein, 6 mL) were homogenized (Model T25 basic; IKA Labortecnik, Selangor, Malaysia) at a speed of 20,000 rpm for 1 min. An aliquot of the emulsion (50 μL) was pipetted from the middle portion of the container at 0 and 10 min after homogenization and subsequently diluted 100-fold using 0.1% SDS solution. The mixture was mixed thoroughly for 10 s using a vortex mixer (G-560E, Vortex-Genie 2, Scientific Industries, Inc., Bohemia, NY, USA). A_{500} of the resulting dispersion was measured using a spectrophotometer (UV-1800, Shimadzu). EAI and ESI were calculated by the following formulae:

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

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

$$\text{ESI (min)} = A_0 \times \Delta t/\Delta A$$

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

9.3.7.3 Foaming properties

Foam expansion (FE) and foam stability (FS) of hydrolysate solutions were determined as described by Shahidi *et al.* (1995) with a slight modification. Hydrolysate solutions (20 mL) with 0.10, 0.25 and 0.50% protein concentrations were transferred into 100-mL cylinder. The solutions were homogenized at 13,400 rpm for 1 min at room temperature. The samples were allowed to stand for 0 and 60 min. FE and FS were then calculated using the following equations:

$$FE\ (\%) = (V_T/V_0)\times 100$$
$$FS\ (\%) = (V_t/V_0)\times 100$$

where V_T is total volume after whipping; V_0 is the original volume before whipping and V_t is total volume after leaving at room temperature for 60 min.

9.3.8 Determination of antioxidative activity

9.3.8.1 DPPH radical scavenging activity

DPPH radical scavenging activity was determined by DPPH assay as described by Binsan *et al.* (2008). Sample (1.5 mL) was added with 1.5 mL of 0.15 mM DPPH in 95% ethanol. The mixture was mixed vigorously and allowed to stand at room temperature in the dark for 30 min. The absorbance of the resulting solution was measured at 517 nm using a UV-1800 spectrophotometer (Shimadzu, Kyoto, Japan). The blank was prepared in the same manner, except that distilled water was used instead of the sample. A standard curve was prepared using Trolox in the range of 10–60 μM. The activity was expressed as μmol Trolox equivalents (TE)/g protein of hydrolysate.

9.3.8.2 ABTS radical scavenging activity

ABTS radical scavenging activity was determined by ABTS assay as described by Binsan *et al.* (2008). The stock solutions included 7.4 mM ABTS solution and 2.6 mM potassium persulfate solution. The working solution was prepared by mixing the two stock solutions in equal quantities and allowing them to

react for 12 h at room temperature in dark. The solution was then diluted by mixing 1 mL ABTS solution with 50 mL methanol in order to obtain an absorbance of 1.1 ± 0.02 units at 734 nm using a UV-1800 spectrophotometer (Shimadzu, Kyoto, Japan). Fresh ABTS solution was prepared for each assay. Sample (150 µL) was mixed with 2850 µL of ABTS solution and the mixture was left at room temperature for 2 h in dark. The absorbance was then measured at 734 nm using a spectrophotometer. The blank was prepared in the same manner except that the distilled water was used instead of the sample. A standard curve of Trolox ranging from 50 to 600 µM was prepared. The activity was expressed as µmol TE/g protein of hydrolysate.

9.3.8.3 Chelating activity on Fe^{2+}

The chelation of Fe^{2+} was measured using the method of Boyer and McCleary (1987) with a slight modification. Diluted sample (4.7 mL) was mixed with 0.1 mL of 2 mM $FeCl_2$ and 0.2 mL of 5 mM ferrozine. The reaction mixture was allowed to stand for 20 min at room temperature. The absorbance was then read at 562 nm. The blank was prepared by using the distilled water instead of the sample. The chelating activity was calculated as follows:

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

where A is A_{562} of sample and B is A_{562} of the blank.

9.3.9 Fractionation and molecular weight distribution of antioxidative peptides from protein hydrolysate

The hydrolysate (20% DH) rendering the highest antioxidative activity was subjected to gel filtration chromatography. The hydrolysate was loaded onto a Sephadex G-25 column (1.6 × 70 cm). The elution was done with 20 mM sodium phosphate buffer (pH 7.0) at a flow rate of 1 mL/min. The 2-mL fractions were collected and their absorbance was read at 220 and 280 nm. The fractions were subjected to analysis for ABTS radical scavenging activity. The fractions with the

maximal ABTS radical scavenging activity were pooled, referred to as “antioxidative peptide fraction”, and used for further study. The molecular weight distribution of antioxidative peptides was determined from the retention time of selected fraction in comparison with those of protein standards including gramicidin (1,880 Da), vitamin B12 (1,355 Da), flavin adenine dinucleotide (829.5 Da) and Gly-Tyr (238.25 Da).

9.3.10 pH and thermal stability of antioxidative peptides

For the pH stability study, the antioxidative peptides fraction was adjusted to different pHs (1, 2, 3, 4, 5, 6, 7, 8, 9, 10 and 11) using 1 M HCl and 1 M NaOH and incubated at room temperature for 30 min. Thereafter, the pHs of the sample were adjusted to pH 7.0. The residual antioxidative activities were determined by ABTS assay.

To determine thermal stability, 5 mL of antioxidative peptide fraction (pH 7.0) were transferred into a screw-cap test tube. The tube was capped tightly and placed in boiling water bath (100 °C) for 0, 15, 30, 60, 90, 120 and 180 min. The treated samples were suddenly cooled in iced water. The sample without incubation (25 °C) was used as the control. The residual antioxidative activities were determined by ABTS assay.

9.3.11 *In vitro* pepsin–pancreatin simulated gastrointestinal (GI) digestion of antioxidative peptides

Simulated GI digestion using an *in vitro* pepsin–pancreatin hydrolysis was carried out according to the method of Cinq-Mars *et al.* (2008) and You *et al.* (2010) with a slight modification. The pH of antioxidative peptide fraction containing 3 mg protein (15 mL) was adjusted to 2.0 with 6 N HCl. Pepsin was then added (E/S 1:35 w/w), and the mixture was incubated with continuous shaking (W350, Memmert, Schwabach, Germany) for 1 h at 37 °C. The pH was then adjusted to 5.3 with 0.9 M $NaHCO_3$ solution and further to pH 7.5 with 6 N NaOH. Pancreatin was

added (E/S 1:25 w/w), and the mixture was further incubated with continuous shaking for 3 h at 37 °C. To terminate the digestion, the solution was submerged in boiling water for 10 min. Then, the GI digest was cooled to room temperature and centrifuged at 5000 ×*g* for 15 min. The supernatant was use for analysis.

To investigate the changes in ABTS radical scavenging activity of peptide digests during the simulated GI digestion, aliquots of GI digests were removed every 30 min during the *in vitro* digestion of totally 3 h.

9.3.12 Protein determination

Protein concentration was determined using the Lowry method (Lowry *et al.*, 1951). Bovine serum albumin was used as a standard.

9.3.13 Statistical analysis

All experiments were performed in triplicate. The data were subjected to Analysis of Variance (ANOVA) and the differences between means were evaluated by Duncan's multiple range test. SPSS statistic program (SPSS 11.0 for Windows, SPSS Inc., Chicago, IL, USA) was used for data analysis.

9.4 Results and Discussion

9.4.1 Solubility of protein hydrolysates with different DHs

The solubilities of hydrolysates from the muscle of ornate threadfin bream with different DHs at various pHs are shown in Table 21. All hydrolysates were soluble over a wide pH range, in which more than 70% solubility was obtained. The hydrolysates were generally soluble in alkaline pH to a greater extent, compared with acidic pH, excepted at pH 3 for hydrolysate with 10% DH. The lowest solubility was found in hydrolysate with 10% DH at pH 5 ($P < 0.05$). The result suggested that proteins or peptides with high molecular weight (MW) remaining after hydrolysis were precipitated at this pH, which was close to the isoelectric point (pI) of

myofibrillar proteins. In general, the degradation of proteins to smaller peptides leads to more soluble products (Gbogouri *et al.*, 2004; Klompong *et al.*, 2007). The smaller peptides from myofibrillar proteins are expected to have proportionally more polar residues, with the ability to form hydrogen bonds with water and augment solubility (Gbogouri *et al.*, 2004). At pH 5 and 9, the solubilities of hydrolysates were increased with increasing DH from 10% to 20% DH ($P < 0.05$). No differences in the solubility of hydrolysate with different DHs were observed at pH 7 ($P > 0.05$). Enzymatic hydrolysis potentially affects the molecular size and hydrophobicity, as well as polar and ionizable groups of protein hydrolysates (Mutilangi *et al.*, 1996). The balance of hydrophilic and hydrophobic forces of peptides is another crucial influence on solubility of protein hydrolysate (Gbogouri *et al.*, 2004; Klompong *et al.*, 2007). Solubility is one of the most important physicochemical and functional properties of protein hydrolysates (Kristinsson and Rasco, 2000; Thiansilakul *et al.*, 2007a). Good solubility of proteins is required in many functional applications, especially for emulsions, foams and gels (Thiansilakul *et al.*, 2007a). Therefore, the differences in solubility of hydrolysates with different DHs might be determined by the size of peptides, the hydrophobic-hydrophilic balance as well as charge group of the peptides produced during hydrolysis process.

Table 21. Solubility of hydrolysates from ornate threadfin bream muscle prepared using pepsin from skipjack tuna with different DHs at various pHs.

pH	DH (%)		
	10	20	30
3	97.8 ± 2.0b[†]B[‡]	92.8 ± 2.5aA	91.4 ± 1.2aA
5	71.2 ± 1.1aA	93.1 ± 0.6aB	92.4 ± 0.5aB
7	97.0 ± 0.5bA	97.5 ± 1.9bA	98.2 ± 1.8bA
9	96.6 ± 0.7bA	99.3 ± 1.0bB	98.1 ± 1.8bAB

Means ± SD (n = 3).

[†] Different small letters in the same column indicate significant differences ($P < 0.05$).

[‡] Different capital letters in the same row indicate significant differences ($P < 0.05$).

9.4.2 Emulsifying properties of protein hydrolysates with different DHs

Emulsion activity index (EAI) and emulsion stability index (ESI) of hydrolysates from ornate threadfin bream muscle with different DHs at various concentrations (0.10, 0.25, and 0.50%) are shown in Table 22. Generally, both EAI and ESI of hydrolysates with different DHs varied particularly when the protein concentration increased ($P < 0.05$). The differences in emulsifying properties of hydrolysates with different DHs might result from the varying intrinsic properties, compositions and conformation. At 0.10% protein, hydrolysate with 10% DH showed the highest EAI and ESI, when compared with those having higher DHs ($P < 0.05$). However, no differences in EAI were found with increasing DH, when hydrolysates at 0.25 and 0.50% were tested ($P > 0.05$). Mutilangi *et al.* (1996) postulated that higher contents of larger MW peptides or more hydrophobic peptides contribute to the stability of the emulsion. On the other hand, the excessive hydrolysis brings about the loss of emulsifying properties (Gbogouri *et al.*, 2004; Klompong *et al.*, 2007; Kristinsson and Rasco, 2000). The peptides with low MW may not be amphiphilic enough to exhibit good emulsifying properties (Chobert *et al.*, 1988). Lin and Chen (2006) proposed that the emulsification process includes two steps: 1) deformation and disruption of droplets which increase the specific surface area of emulsion and 2) the stabilization of this newly formed interface by emulsifier or surfactant. The hydrolysate with 10% DH could arrange at the interface and stabilize the oil droplet to a higher extent. With the same DH, EAI of hydrolysates decreased with increasing concentrations ($P < 0.05$). With 20 and 30% DH, no differences in ESI were noticeable as the hydrolysate concentrations were above 0.25% ($P > 0.05$). At low protein concentrations, protein adsorption at the oil–water interface is diffusion controlled. At high protein concentration, activation energy barrier does not allow protein migration to take place in a diffusion dependent manner, leading to the accumulation of proteins in the aqueous phase (Lawal, 2004; Thiansilakul et al., 2007a). Thus, proteins or peptides were most likely localized in the aqueous phase and the lower amount of proteins or peptides were migrated to interface (Thiansilakul *et al.*, 2007a). The increase in protein-protein interaction resulted in the lower protein concentration at the interface, in which thinner film stabilizing the oil droplet is

formed. Therefore, emulsifying properties of hydrolysate were governed by the molecular properties, particularly the size of peptides and concentration used.

Table 22. Emulsifying and foaming properties of hydrolysates from ornate threadfin bream muscle prepared using pepsin from skipjack tuna with different DHs at various protein concentrations.

Hydrolysate concentrations (% protein)	DH (%)	EAI (m^2/g)	ESI (min)	FE (%)	FS (%)
0.10	10	116 ± 2.6c[†]C[‡]	25.8 ± 2.2bC	128 ± 3.1bA	69 ± 5.3bA
	20	109 ± 2.3bC	15.2 ± 0.6aA	126 ± 2.4bA	57 ± 2.5aA
	30	102 ± 2.9aC	16.1 ± 0.4aA	120 ± 2.3aA	55 ± 2.9aA
0.25	10	58.6 ± 1.5bB	18.4 ± 2.3bB	128 ± 3.0bA	75 ± 3.2bAB
	20	54.8 ± 1.0aB	18.5 ± 1.0bB	127 ± 2.1bA	65 ± 5.2abAB
	30	55.0 ± 0.2aB	17.5 ± 0.7bB	118 ± 3.3aA	60 ± 6.1aA
0.50	10	29.9 ± 2.3aA	14.1 ± 0.5aA	131 ± 4.5bA	78 ± 2.5cB
	20	30.3 ± 0.3aA	18.6 ± 2.5bB	126 ± 4.5abA	70 ± 4.8bB
	30	30.1 ± 1.1aA	17.8 ± 0.8bB	122 ± 4.0aA	61 ± 2.3aA

Means ± SD (n = 3). [†] Different small letters in the same column within the same hydrolysate concentration indicate significant differences ($P < 0.05$). [‡] Different capital letters in the same column within the same DH indicate significant differences ($P < 0.05$).

9.4.3 Foaming properties of protein hydrolysates with different DHs

Foaming properties are physicochemical characteristics of proteins to form and stabilize foams (Thiansilakul *et al.*, 2007a). Foam expansion (FE) and foam stability (FS) of hydrolysates from ornate threadfin bream muscle with different DHs at various concentrations (0.10, 0.25, and 0.50%) are shown in Table 22. At the same concentration of hydrolysate used, the slight decreases in FE were observed when DH of hydrolysate increased ($P < 0.05$). This was possibly due to the lower alignment of small peptides at air-water interface (Nalinanon *et al.*, 2008a). With the same DH, protein concentrations had no impact on FE ($P > 0.05$). Foam formation is governed by three factors, including transportation, penetration and reorganization of molecules at the air–water interface (Klompong *et al.*, 2007). In general, proteins, which rapidly

adsorb at the newly-created air–liquid interface during bubbling and undergo unfolding and molecular rearrangement at the interface, exhibit better foaming ability than proteins that adsorb slowly and resist unfolding at the interface (Damodaran, 1997). FE after whipping for 60 min was monitored to indicate the FS of protein hydrolysates. FS was slightly decreased with increasing DH of hydrolysate. It was suggested that low MW peptides could not maintain well-ordered orientation of the molecule at the interface. However, at the same DH, FS of hydrolysate was improved by increasing concentration ($P < 0.05$). Lawal (2004) postulated that an increase in foam stability with increasing concentration was a result of formation of stiffer foams. FS is enhanced by flexible protein domains, which enhance viscosity of the aqueous phase, protein concentration and film thickness (Phillips *et al.*, 1994). Zayas (1997) reported that the properties of proteins, which enable them to form stable films in foam, are affected by the molecular configuration of proteins, their intermolecular bonds, and the content and disposition of hydrophobic residues.

9.4.4 Antioxidative activities of protein hydrolysates with different DHs

Antioxidative activities, as determined by ABTS, DPPH and Fe^{2+} chelating assays of hydrolysates with different DHs are shown in Table 23. The differences in activities determined by both assays were observed among protein hydrolysates with different DHs. The highest ABTS and DPPH radical scavenging activities were found in hydrolysates with 20% DH (159.07 and 8.58 μmol TE/g protein, respectively) ($P < 0.05$). ABTS and DPPH radical scavenging activities of protein hydrolysates with 10% DH were 150.33 and 3.15 μmol TE/g protein, respectively, whereas that of 30% DH had the activities of 150.98 and 6.21 μmol TE/g protein, respectively. Thus peptides in hydrolysates with various DHs might scavenge two different radicals, ABTS and DPPH, differently. Generally, 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 (Khantaphant and Benjakul, 2008). ABTS assay is an excellent tool for determining the antioxidant activity of hydrogen-donating antioxidants (scavengers of aqueous phase radicals) and of chain breaking

antioxidants (scavenger of lipid peroxyl radicals) (Binsan *et al.*, 2008). $ABTS^{\bullet+}$ is a relatively stable radical and is readily reduced by antioxidant (Klompong *et al.*, 2009; Miller *et al.*, 1993). With high ABTS radical scavenging activity, it was postulated that antioxidative compounds were most likely hydrophilic. DPPH radical scavenging activity is generally used to determine hydrogen donating ability of protein hydrolysates (Thiansilakul *et al.*, 2007b; Wu *et al.*, 2003). DPPH is a stable free radical that shows the maximal absorbance at 517 nm in ethanol. When DPPH encounters the proton-donating substance, the radical would be scavenged as visualized by changing color from purple to yellow and the absorbance is reduced (Shimada *et al.*, 1992). The result suggested that the hydrolysates contained the amino acids or peptides that were electron donors and could react with free radicals to convert them to more stable products and terminate the radical chain reaction.

As the DH increased, chelating activity on Fe^{2+} increased ($P < 0.05$). Hydrolysates with 30% DH exhibited the highest Fe^{2+} chelating activity (58.1%). The chelation of Fe^{2+} was used to determine the ability of hydrolysates in metal chelating. Ferrozine quantitatively forms complexes with Fe^{2+} ion. In the presence of chelating agents, the complex formation is disrupted, resulting in the decrease in colour formation (Thiansilakul *et al.*, 2007b). The results indicated that hydrolysates had a potential chelating ability towards iron. Therefore, hydrolysates can be used in food system to prevent the lipid oxidation via radical scavenging activity and metal chelating ability.

Table 23. Antioxidative activities of hydrolysates from ornate threadfin bream muscle prepared using pepsin from skipjack tuna with different DHs as determined by various assays

Activities tested	DH (%)		
	10	20	30
ABTS (μmol TE/g protein)	150.33±0.82a†	159.07±1.01b	150.98±0.74a
DPPH (μmol TE/g protein)	3.15±0.35a	8.58±0.24c	6.21±0.12b
Chelating activity (%)	45.92±1.72a	51.33±0.50b	58.11±0.59c

TE: Trolox equivalents. Mean ± SD (n = 3). † Different letters in the same row indicate significant differences ($P < 0.05$).

9.4.5 Fractionation and molecular weight distribution of antioxidative peptides from protein hydrolysate

Hydrolysates from the muscle of ornate threadfin bream with 20% DH prepared using the pepsin from skipjack tuna, which showed the highest radical scavenging activity, was fractionated using Sephadex-G25 gel filtration chromatography. A_{220} was used to monitor peptide bonds, whereas A_{280} was the parameter representing peptides, proteins or amino acids with aromatic rings (Figure 37A). A distinct peak of A_{280} was observed in the hydrolysate, reflecting the presence of proteins or peptides with high and low MW. The hydrolysates had two peaks of A_{220}, indicating the presence of peptide bonds in the hydrolysate. The first peak of A_{220} was found in the same fraction of the A_{280} peak. Low A_{280} was detected in the second A_{220} peak, indicating high proportion of low MW peptides in the hydrolysate.

As shown in Figure 37B, the fraction containing peptides with a MW of 1.3 kDa of hydrolysate prepared using skipjack tuna pepsin showed the highest ABTS radical scavenging activity. The activity peak was located at the same position of the second A_{220} peak, indicating that low MW peptides contributed to the antioxidative activity. The result suggested that hydrolysate most likely contained the certain peptides that were electron donors or could scavenge free radicals to terminate the radical chain reaction. Fractions no. 49-53 showing the highest ABTS radical scavenging activity were pooled and used as a source of antioxidative peptides.

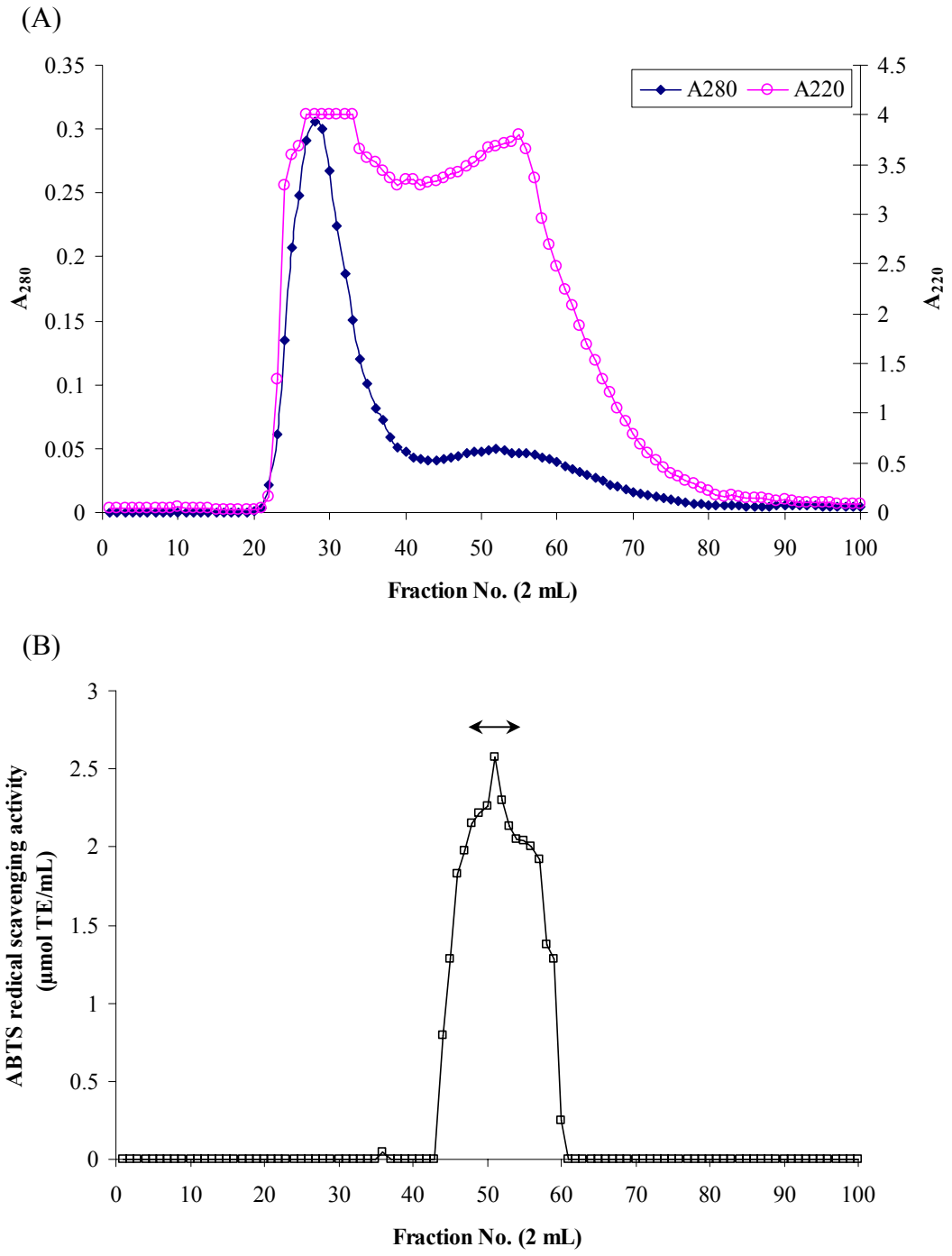

Figure 37. Separation of antioxidative peptides from hydrolysate of ornate threadfin bream muscle prepared using pepsin from skipjack tuna. (A): A_{220} and A_{280}; (B): ABTS radical scavenging activity. (↔): pooled fractions.

9.4.6 pH and thermal stability of antioxidative peptides

The influences of pH on the stability of antioxidative peptides fractionated by Sephadex G-25 column chromatography are depicted in Figure 38A. ABTS radical scavenging activity of the antioxidative peptide fraction remained constant when subjected to the pH range of 1–10. At pH 11, ABTS radical scavenging activity slightly decreased ($P < 0.05$). The results suggested that antioxidative peptides exhibiting ABTS radical scavenging activity might loss their activity to some extent at the extremely alkaline pHs. Due to the stability over a wide pH range, antioxidative peptides from the muscle of ornate threadfin bream had a potential in applications in any food system with a harsh pH.

Thermal stability of antioxidative peptide fraction as monitored by ABTS radical scavenging activities is shown in Figure 38B. ABTS radical scavenging activities of antioxidative peptide fraction was quite stable when heated at 100 °C up to 180 min, where activities of more than 97% were retained. A small loss in ABTS radical activity might be due to either degradation or aggregation of some antioxidative peptides, caused by heat treatment. In general, proteins were vulnerable to heat treatment, leading to the aggregation of protein and the exposure of hydrophobic domain (Sikorski and Naczk, 1981). Nevertheless, peptides with smaller sizes were more stable to aggregation at high temperature (Zayas, 1997). Peptides derived from many protein sources with increased hydrophobicity have been reported to correlate with antioxidative properties (Chen *et al.*, 1996; Faithong *et al.*, 2010). The result indicated that peptides with low MW were most likely stable after heat treatment. Therefore, the antioxidative peptides can be incorporated in cooked food system without significant loss of their antioxidant activity.

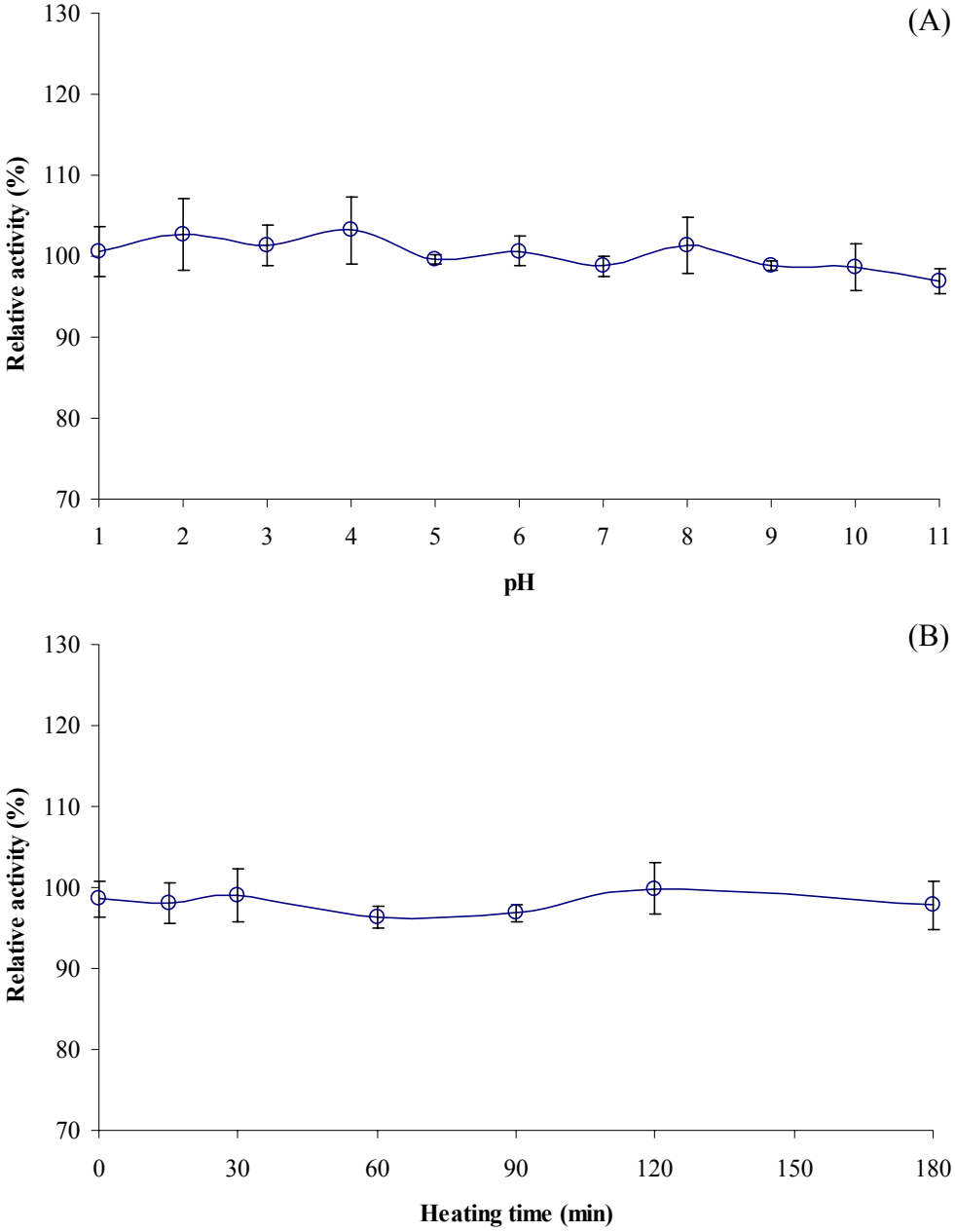

Figure 38. pH (A) and thermal (B) stabilities of antioxidative peptides from hydrolysate of ornate threadfin bream using pepsin from skipjack tuna fractioned by Sephadex G-25 column chromatography. Bars represent the standard deviation (n = 3).

9.4.7 *In vitro* pepsin–pancreatin simulated GI digestion of antioxidative peptides

An *in vitro* pepsin–pancreatin hydrolysis simulating GI digestion was used to illustrate its impact on ABTS radical scavenging activity of antioxidative peptides (Sephadex G-25 fraction) from protein hydrolysate of ornate threadfin bream prepared using skipjack tuna pepsin (Figure 39). Pepsin digestion for 1 h did not alter ABTS radical scavenging activity ($P > 0.05$). This might be due to the cleavage limitation of pepsin on antioxidative peptides, which were previously digested by skipjack tuna pepsin. As a result, no degradation or generation of new antioxidative peptides took place. After further digestion by pancreatin for 30-60 min, ABTS radical scavenging activity was increased by about 7-12%. It was suggested that antioxidative peptides were modified by pancreatin digestion to enhance their ABTS radical scavenging activity. In addition, the increase of hydrophilic property of GI digests after pancreatin treatment has been reported to favor trapping $ABTS^{\bullet+}$ radical of loach protein hydrolysate (You *et al.*, 2010). Additionally, You *et al.* (2010) reported that GI digests had the increased reducing power, chelating ability of Cu^{2+} and hydroxyl radical scavenging activities by 77, 12 and 12%, respectively. Therefore, antioxidative peptides were more likely stable in real digestion system after ingestion in both stomach and intestine, which have high proteolytic activity under acidic and alkaline pH, respectively.

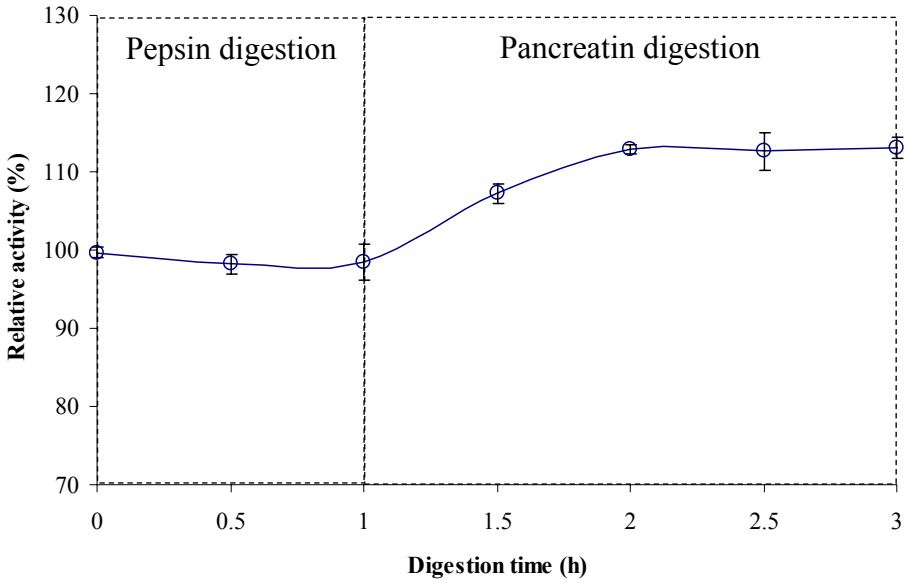

Figure 39. Changes in ABTS radical scavenging activity during sequential *in vitro* digestion of antioxidative peptides from hydrolysate of ornate threadfin bream using pepsin from skipjack tuna fractioned by Sephadex G-25 column chromatography. The first stage: from 0 to 1.0 h, digested by pepsin; the second stage: from 1.0 to 3.0 h, digested by pancreatin. Bars represent the standard deviation (n = 3).

9.5 Conclusion

Production of protein hydrolysates from ornate threadfin bream muscle, exerting good functionalities and antioxidative activities, could be achieved by skipjack tuna pepsin hydrolysis. Based on pH and thermal stability as well as GI digestion, antioxidative peptides in protein hydroysate can be incorporated as the multifunctional ingredient into foods.

CHAPTER 10

SUMMARY AND FUTURE WORKS

10.1 Summary

1. Tuna pepsins, including albacore tuna, skipjack tuna and tongol tuna, with optimal activity at pH 2 and 50 °C were strongly inhibited by pepstatin A, SDS, and cysteine, when hemoglobin was used as a substrate.
2. Pepsinogen from the stomach of albacore tuna was purified to homogeneity, based on the native-PAGE and zymography. It was converted to the corresponding pepsin at pH 2 through an intermediate form (MW≈36.8 kDa) and the complete activation was observed after 60 min. Resultant pepsin exhibited the proteolytic activity similar to other fish pepsins, particularly from tropical fish.
3. Purified pepsins 1 and 2 from the stomach of skipjack tuna had high affinity and hydrolytic activity toward hemoglobin. Both pepsins effectively hydrolyzed bovine serum albumin, egg white, natural actomyosin from brownstripe red snapper muscle and acid-solubilized collagen from arabesque greenling skin.
4. The optimum condition for partitioning of SE and ASE from the stomach of albacore tuna was 25% PEG1000-20% $MgSO_4$ and 15% PEG2000-15% $MgSO_4$, respectively. After phase separation, protein with MW of 32.7 kDa became dominant and the protease was gradually lost their activity in time-dependent manner. Instability of protease was more pronounced in higher storage temperature.
5. Collagen extraction from threadfin bream skin could be achieved by soaking the prepared skin in 0.5 M acetic acid containing albacore pepsin at a level of 10 units/g defatted skin for 12 h. Albacore pepsin exhibited the comparable extraction efficiency to porcine pepsin and no detrimental effect on the integrity of resulting collagen was observed.

6. Efficiency in collagen extraction from the skin of unicorn leatherjacket could be enhanced by 8 folds when pepsin from albacore tuna at 20 units/g of residual skin was used. PSC obtained exhibited similar molecular properties to ASC.
7. Use of albacore tuna pepsin could recover residual collagen from acid treated skin of arabesque greenling, thereby increasing total extraction yield. However, pepsin might alter collagen structure, which governed the molecular properties of resultant collagen to some degree.
8. Type I collagen from the skin ornate threadfin bream contained $[\alpha1(I)]_2\alpha2(I)$ as the dominant component with the co-presence of $\alpha1(I)\alpha2(I)\alpha3(I)$. Pepsin from the stomach of skipjack tuna showed greater ability to cleave the cross-linked components without the cleavage of major components of collagen, whereas porcine pepsin more likely hydrolyzed all components, especially at high temperature.
9. Production of protein hydrolysates from ornate threadfin bream muscle, exerting good functionalities and antioxidative activities, could be achieved by skipjack tuna pepsin hydrolysis. Based on pH and thermal stabilities as well as GI digestion, antioxidative peptides in protein hydroysate can be incorporated as the multifunctional ingredient into foods.

10.2 Future works

1. Although ATPS can be effectively used to recover and purify protease from tuna stomach, the method to separate pepsin from PEG without the loss in activity should be developed.
2. Fish pepsin of other species recovered from processing by-products should be studied.
3. Based on biochemical properties, the application of tuna pepsin in various food products should be more intensively investigated.
4. The stability and preservation of purified tuna pepsin should be studied.
5. Impact of freshness of fish on the extraction and characteristics of collagen, especially pepsin solubilized collagen, should be investigated.

REFERENCES

Abad-Zapatero, C., Rydel, T.J. and Erickson, J. 1990. Revised 2.3 Å structure of porcine pepsin: evidence for a flexible subdomain. Proteins. 8: 62-81.

Abu Erreish, G.M. and Peanasky, R.J. 1974. Pepsin inhibitors from *Ascaris lumbricoides*. Isolation, purification, and some properties. J. Biol. Chem. 249: 1558-1565.

Abuereish, G.M. 1998. Pepsin inhibitor from roots of *Anchusa strigosa*. Phytochemistry. 48: 217-221.

Al-Janabi, J., Hartsuck, J.A. and Tang, J. 1972. Kinetics and mechanism of pepsinogen activation. J. Biol. Chem. 247: 4628-4632.

Albertsson, P.A. 1958. Partition of proteins in liquid polymer-polymer two-phase systems. Nature. 182: 709-711.

Albertsson, P.A. 1986. Aqueous polymer-phase systems. *In* Partition of cell particles and macromolecules. 3rd Ed. p. 8-38. John Wiley & Son Inc. . New York.

Albertsson, P.A. and Tjerneld, F. 1994. Phase diagram. *In* Methods in enzymology. (Walter, H. and Johansson, G., eds.). p. 161-226. Academic Press. San Diego.

An, H., Seymour, T.A., Wu, J. and Morrissey, M.T. 1994. Assay systems and characterization of Pacific whiting (*Merluccius productus*) protease. J. Food Sci. 59: 277-281.

Andreeva, N.S., Zdanov, A.S., Gustchina, A.E. and Fedorov, A.S. 1984. Structure of ethanol-inhibited porcine pepsin at 2-Å resolution and binding of the methyl ester of phenylalanyl-diiodotyrosine to the enzyme. J. Biol. Chem. 259: 11353-11365.

Antov, M.G., Pericin, D.M. and Dasic, M.G. 2006. Aqueous two-phase partitioning of xylanase produced by solid-state cultivation of Polyporus squamosus. Process Biochem. 41: 232-235.

Arnoštová, H., Kucerová, Z., Tišlerová, I., Trnka, T. and Tichá, M. 2001. Affinity chromatography of porcine pepsin on different types of immobilized 3,5-diiodo-L-tyrosine. J. Chromatogr. A. 911: 211-216.

Arunchalam, K. and Haard, N.F. 1985. Isolation and characterization of pepsin from polar cod (*Boreogadus saida*). Comp. Biochem. Physiol. B. 80: 467-473.

Bae, I., Osatomi, K., Yoshida, A., Osako, K., Yamaguchi, A. and Hara, K. 2008. Biochemical properties of acid-soluble collagens extracted from the skins of underutilised fishes. Food Chem. 108: 49-54.

Bailey, A.J., Paul, R.G. and Knott, L. 1998. Mechanisms of maturation and ageing of collagen. Mech. Ageing Dev. 106: 1-56.

Bateman, K.S., Chernaia, M.M., Tarasova, N.I. and James, M.N.G. 1998. Crystal structure of human pepsinogen A. Adv. Exp. Med. Biol. 436: 259-263.

Baudyš, M. and Kostka, V. 1983. Covalent structure of chicken pepsinogen. Eur. J. Biochem. 136: 89-99.

Baudyš, M., Erdene, T.G., Kostka, V., Pavlík, M. and Foltmann, B. 1988. Comparison between prochymosin and pepsinogen from lamb and calf. Comp. Biochem. Physiol. B. 89: 385-391.

Bejosano, F.P. and Corke, H. 1999. Properties of protein concentrates and hydrolysates from *Amaranthus* and Buckwheat. Ind. Crop. Prod. 10: 175-183.

Belitz, H.-D., Grosch, W. and Schieberle, P. 2004. Meat. *In* Food chemistry. 3rd Ed. (Belitz, H.-D. *et al.*, eds.). p. 566-616. Springer-Verlag. Berlin.

Benjakul, S. and Morrissey, M.T. 1997. Protein hydrolysates from Pacific whiting solid wastes. J. Agric. Food Chem. 45: 3423-3430.

Benjakul, S., Seymour, T.A., Morrissey, M.T. and An, H. 1997. Physicochemical changes in Pacific whiting muscle proteins during iced storage. J. Food Sci. 62: 729-733.

Benjakul, S., Visessanguan, W. and Tanaka, M. 2003. Partial purification and characterization of trimethylamine-N-oxide demethylase from lizardfish kidney. Comp. Biochem. Physiol. B. 135: 359-371.

Benjakul, S., Visessanguan, W., Thongkaew, C. and Tanaka, M. 2005. Effect of frozen storage on chemical and gel-forming properties of fish commonly used for surimi production in Thailand. Food Hydrocolloid. 19: 197-207.

Benjakul, S., Thiansilakul, Y., Visessanguan, W., Roytrakul, S., Kishimura, H., Prodpran, T. and Meesane, J. 2010. Extraction and characterisation of pepsin-solubilised collagens from the skin of bigeye snapper (*Priacanthus tayenus* and *Priacanthus macracanthus*). J. Sci. Food Agric. 90: 132-138.

Bensch, M., Selbach, B. and Hubbuch, J. 2007. High throughput screening techniques in downstream processing: Preparation, characterization and optimization of aqueous two-phase systems. Chem. Eng. Sci. 62: 2011-2021.

Binsan, W., Benjakul, S., Visessanguan, W., Roytrakul, S., Tanaka, M. and Kishimura, H. 2008. Antioxidative activity of Mungoong, an extract paste, from the cephalothorax of white shrimp (*Litopenaeus vannamei*). Food Chem. 106: 185-193.

Boeris, V., Spelzini, D., Farruggia, B. and Picó, G. 2009. Aqueous two-phase extraction and polyelectrolyte precipitation combination: A simple and economically technologies for pepsin isolation from bovine abomasum homogenate. Process Biochem. 44: 1260-1264.

Bohak, Z. 1973. The kinetics of the conversion of chicken pepsinogen to chicken pepsin. Eur. J. Biochem. 32: 547-554.

Bougatef, A., Balti, R., Ben Zaied, S., Souissi, N. and Nasri, M. 2008. Pepsinogen and pepsin from the stomach of smooth hound (*Mustelus mustelus*): Purification, characterization and amino acid terminal sequences. Food Chem. 107: 777-784.

Boyer, R. 2006. Enzyme II: cofactors, regulation and catalytic RNA. *In* Concepts in biochemistry. 3rd Ed. (Boyer, R., ed.). p. 178. John Willey & Son, Inc.

Boyer, R.F. and McCleary, C.J. 1987. Superoxide ion as a primary reductant in ascorbate-mediated ferritin iron release. Free Radical Biol. Med. 3: 389-395.

Brewer, P., Helbig, N. and Haard, N.F. 1984. Atlantic cod pepsin – characterization and use as a rennet substitute. Can. Inst. Food Sci. Technol. J. 17: 038-043.

Burghagen, M. 1999. Collagen. *In* Food chemistry. 2nd Ed. (Belitz, H. D. and Grosch, W., eds.). p. 540-547. Springer-verlag Berlin Heidelberg. Berlin.

Castillo-Yañez, F.J., Pacheco-Aguilar, R., García-Carreño, F.L. and Toro, M.d.l.A.N.-D. 2004. Characterization of acidic proteolytic enzymes from Monterey sardine (*Sardinops sagax caerulea*) viscera. Food Chem. 85: 343-350.

Chen, H.M., Muramoto, K., Yamauchi, F. and Nokihara, K. 1996. Antioxidant activity of designed peptides based on the antioxidative peptide isolated from digests of a soybean protein. J. Agric. Food Chem. 44: 2619-2623.

Chen, W.-Q., Cao, M.-J., Yoshida, A., Liu, G.-M., Weng, W.-Y., Sun, L.-C. and Su, W.-J. 2009. Study on pepsinogens and pepsins from snakehead (*Channa argus*). J. Agric. Food Chem. 57: 10972-10978.

Chobert, J.M., Bertrand-Harb, C. and Nicolas, M.G. 1988. Solubility and emulsifying properties of caseins and whey proteins modified enzymatically by trypsin. J. Agric. Food Chem. 36: 883-892.

Chomarat, N., Robert, L., Seris, J.L. and Kern, P. 1994. Comparative efficiency of pepsin and proctase for the preparation of bovine skin gelatin. Enzyme and Microbial Technology. 16: 756-760.

Chouyyok, W., Wongmongkol, N., Siwarungson, N. and Prichanont, S. 2005. Extraction of alkaline protease using an aqueous two-phase system from cell free Bacillus subtilis TISTR 25 fermentation broth. Process Biochem. 40: 3514-3518.

Chow, R.B. and Kassel, B. 1968. Bovine pepsinogen and pepsin. I. Isolation, purification, and some properties of the pepsinogen. J. Biol. Chem. 243: 1718-1724.

Christensen, K.A., Pedersen, V.B. and Foltmann, B. 1977. Identification of an enzymatically active intermediate in the activation of porcine pepsinogen. FEBS Lett. 76: 214-218.

Cinq-Mars, C.D., Hu, C., Kitts, D.D. and Li-Chan, E.C.Y. 2008. Investigations into inhibitor type and mode, simulated gastrointestinal digestion, and cell transport of the angiotensin I-converting enzyme-inhibitory peptides in Pacific hake (*Merluccius productus*) fillet hydrolysate. J. Agric. Food Chem. 56: 410-419.

Cooper, J.B., Khan, G., Taylor, G., Tickle, I.J. and Blundell, T.L. 1990. X-ray analyses of aspartic proteinases. II. Three-dimensional structure of the hexagonal crystal form of porcine pepsin at 2.3 Å resolution. J. Mol. Biol. 214: 199-222.

Cunningham, M. and Tang, J. 1976. Purification and properties of cathepsin D from porcine spleen. J. Biol. Chem. 251: 4528-4536.

Damodaran, S. 1997. Protein-stabilized foams and emulsions. *In* Food proteins and their applications. (Damodaran, S. and Paraf, A., eds.). p. 57-110. Marcel Dekker Inc. New York.

Davies, D.R. 1990. The structure and function of the aspartic proteinases. Annu. Rev. Biophys. Biophys. Chem. 19: 189-215.

del-Val, M.I. and Otero, C. 2003. Biphasic aqueous media containing polyethylene glycol for the enzymatic synthesis of oligosaccharides from lactose. Enzyme Microb. Technol. 33: 118-126.

Department of Foreign Trade. 2007. Canned tuna (HS 1604140100) (online). Available: www.dft.moc.go.th. (4 Jul 2007)

Department of Foreign Trade. 2009. Canned tuna situation (HS 16041410001) (online). Available: http://www.dft.moc.go.th. (17 Dec 2009)

Diamond, A.D. and Hsu, J.T. 1992. Aqueous two-phase systems for biomolecule separation. Adv. Biochem. Eng. Biotechnol. 47: 89-135.

Díaz-López, M., Moyano-López, F.J., Alarcón-López, F.J., García-Carreño, F.L. and Navarrete del Toro, M.A. 1998. Characterization of fish acid proteases by substrate-gel electrophoresis. Comp. Biochem. Physiol. B. 121: 369-377.

Drake, M.P., Davison, P.F., Bump, S. and Schmitt, F.O. 1966. Action of proteolytic enzymes on tropocollagen and insoluble collagen. Biochemistry. 5: 301-312.

Duan, R., Zhang, J., Du, X., Yao, X. and Konno, K. 2009. Properties of collagen from skin, scale and bone of carp (*Cyprinus carpio*). Food Chem. 112: 702-706.

Estela da Silva, M. and Teixeira Franco, T. 2000. Purification of soybean peroxidase (*Glycine max*) by metal affinity partitioning in aqueous two-phase systems. J. Chromatogr. B. 743: 287-294.

Faithong, N., Benjakul, S., Phatcharat, S. and Binsan, W. 2010. Chemical composition and antioxidative activity of Thai traditional fermented shrimp and krill products. Food Chem. 119: 133-140.

Farruggia, B., Rigatuso, R., Capezio, L., Diez, V. and Picó, G. 2004. Influence of high concentration monovalent cations on the protein partitioning in polyethyleneglycol 1500-phosphate aqueous two-phase systems. J. Chromatogr. B. 809: 301-306.

Fernandez Lahore, H.M., Miranda, M.V., Fraile, E.R., Biscoglio de Jimenez Bonino, M.J. and Cascone, O. 1995. Partition behaviour and purification of a *Mucor bacilliformis* acid protease in aqueous two-phase systems. Process Biochem. 30: 615-621.

Foegeding, E.A., Lanier, T.C. and Hultin, H.O. 1996. Characteristics of edible muscle tissues. *In* Food chemistry. 3rd Ed. (Fennema, O. R., ed.). p. 902–906. Marcel Dekker Inc. New York.

Fox, P.F., Whitaker, J.R. and O'Leary, P.A. 1977. Isolation and characterization of sheep pepsin. Biochem. J. 161: 389-398.

Frýdlová, J., Kucerová, Z. and Tichá, M. 2004. Affinity chromatography of porcine pepsin and pepsinogen using immobilized ligands derived from the specific substrate for this enzyme. J. Chromatogr. B. 800: 109-114.

Fujinaga, M., Chernaia, M.M., Tarasova, N.I., Mosimann, S.C. and James, M.N.G. 1995. Crystal structure of human pepsin and its complex with pepstatin. Protein Sci. 4: 960-972.

Fusek, M. and Větvička, V. 1995. Aspartic proteinases: Physiology and pathology. CRC Press. New York.

Gautam, S. and Simon, L. 2006. Partitioning of β-glucosidase from *Trichoderma reesei* in poly(ethylene glycol) and potassium phosphate aqueous two-phase systems: Influence of pH and temperature. Biochem. Eng. J. 30: 104-108.

Gbogouri, G.A., Linder, M., Fanni, J. and Parmentier, M. 2004. Influence of hydrolysis degree on the functional properties of salmon byproducts hydrolysates. J. Food Sci. 69: 615-622.

Gildberg, A. and Raa, J. 1983. Purification and characterization of pepsins from the arctic fish capelin (*Mallotus villosus*). Comp. Biochem. Physiol. A. 75: 337-342.

Gildberg, A. 1987. Perification and characterization of cathepsin D from the digestive gland of the pelagic squid *Todarodes sagittatus*. J. Sci. Food Agric. 39: 85-94.

Gildberg, A. 1988. Aspartic proteinases in fishes and aquatic invertebrates. Comp. Biochem. Physiol. B. 91: 425-435.

Gildberg, A., Olsen, R.L. and Bjarnason, J.B. 1990. Catalytic properties and chemical composition of pepsins from Atlantic cod (*Gadus morhua*). Comp. Biochem. Physiol. B. 96: 323-330.

Giraud-Guille, M.-M., Besseau, L., Chopin, C., Durand, P. and Herbage, D. 2000. Structural aspects of fish skin collagen which forms ordered arrays via liquid crystalline states. Biomaterials. 21: 899-906.

Gu, T. 2000. Liquid-liquid partitioning methods for bioseparations. *In* Handbook of bioseparations. Vol. 2. (Ahuja, S., ed.). p. 329-363. Academic Press. Sandiego.

Guerard, F. and Le Gal, Y. 1987. Characterization of a chymosin-like pepsin from the dogfish *Scyliorhinus canicula*. Comp. Biochem. Physiol. B. 88: 823-827.

Gündüz, U. and Korkmaz, K. 2000. Bovine serum albumin partitioning in an aqueous two-phase system: Effect of pH and sodium chloride concentration. J. Chromatogr. B. 743: 255-258.

Haard, N.F., Helbig, N. and Feltham, L.A.W. 1981. The temperature characteristics of pepsin from two stock of American salmon (*Osmerus mordax*). Proc. Workshop of the Labrador Coastal Offshore Region. Newfoundland Institute of Cold Ocean Science. 174-196.

Haard, N.F. 1986. Atlantic cod gastric protease. Characterization with casein and milk substrate and influence of Sepharose immobilization on salt activation, temperature characteristics and milk clotting reaction. J. Food Sci. 51: 313-316.

Haard, N.F. 1994. Protein hydrolysis in seafoods. *In* Seafoods: Chemistry, processing technology and quality. (Shahidi, F. and Botta, J. R., eds.). p. 10-33. Chapman & Hall. UK.

Han, J.H. and Lee, C.H. 1997. Effects of salts and poly(ethylene glycol)-palmitate on the partitioning of proteins and *Bacillus subtilis* neutral protease in aqueous two-phase systems. Colloids Surf. B. Biointerfaces. 9: 109-116.

Harboe, M., Andersen, P.M. and Foltmann, B. 1974. The activation of bovine pepsinogen. Sequence of the peptides released, identification of a pepsin inhibitor. J. Biol. Chem. 249: 4487-4494.

Hartsuck, J.A., Koelsch, G. and Remington, S.J. 1992. The high-resolution crystal structure of porcine pepsinogen. Proteins. 13: 1-25.

He, G.Q., Zhang, X.Y., Tang, X.J., Chen, Q.H. and Ruan, H. 2005. Partitioning and purification of extracellular p-1,3-1,4-glucanase in aqueous two-phase systems. J. Zhejiang Univ. SCI. 6 B: 825-831.

Hirasawa, A., Athauda, S.B.P. and Takahashi, K. 1996. Purification and characterization of turtle pepsinogen and pepsin. J. Biochem. 120: 407-414.

Huang, J.S., Huang, S.S. and Tang, J. 1979. Cathepsin D isozymes from porcine spleens. Large scale purification and polypeptide chain arrangements. J. Biol. Chem. 254: 11405-11417.

Huddleston, J.G., Ottomar, K.W., Ngonyani, D.M. and Lyddiatt, A. 1991. Influence of system and molecular parameters upon fractionation of intracellular proteins from Saccharomyces by aqueous two-phase partition. Enzyme Microb. Technol. 13: 24-32.

Ikoma, T., Kobayashi, H., Tanaka, J., Walsh, D. and Mann, S. 2003. Physical properties of type I collagen extracted from fish scales of *Pagrus major* and *Oreochromis niloticas*. Int. J. Biol. Macromol. 32: 199-204.

Imelio, N., Marini, A., Spelzini, D., Picó, G. and Farruggia, B. 2008. Pepsin extraction from bovine stomach using aqueous two-phase systems: Molecular mechanism and influence of homogenate mass and phase volume ratio. J. Chromatogr. B. 873: 133-138.

James, M.N.G. and Sielecki, A.R. 1986. Molecular structure of an aspartic proteinase zymogen, porcine pepsinogen, at 1.8 Å resolution. Nature. 319: 33-38.

Je, J.Y., Kim, S.Y. and Kim, S.K. 2005. Preparation and antioxidative activity of hoki frame protein hydrolysate using ultrafiltration membranes. Eur. Food Res. Technol. 221: 157-162.

Jekel, P.A., Weijer, W.J. and Beintema, J.J. 1983. Use of endoproteinase Lys-C from *Lysobacter enzymogenes* in protein sequence analysis. Anal. Biochem. 134: 347-354.

Jongjareonrak, A., Benjakul, S., Visessanguan, W., Nagai, T. and Tanaka, M. 2005. Isolation and characterisation of acid and pepsin-solubilised collagens from the skin of brownstripe red snapper (*Lutjanus vitta*). Food Chem. 93: 475-484.

Jung, W.K. and Kim, S.K. 2007. Calcium-binding peptide derived from pepsinolytic hydrolysates of hoki (*Johnius belengerii*) frame. Eur. Food Res. Technol. 224: 763-767.

Kageyama, T. and Takahashi, K. 1980. Monkey pepsinogens and pepsins. Monkey pepsinogens and pepsins. V. Purification, Characterization, and amino-terminal sequence determination of crab-eating monkey pepsinogens and pepsins. J. Biochem. 88: 635-645.

Kageyama, T. and Takahashi, K. 1983. Occurrence of two different pathways in the activation of porcine pepsinogen to pepsin. J. Biochem. 93: 743-754.

Kageyama, T. and Takahashi, K. 1986. The complete amino acid sequence of monkey pepsinogen A. J. Biol. Chem. 261: 4395-4405.

Kageyama, T., Tanabe, K. and Koiwai, O. 1990. Structure and development of rabbit pepsinogens: Stage-specific zymogens, nucleotide sequences of cDNAs,

molecular evolution, and gene expression during development. J. Biol. Chem. 265: 17031-17038.

Kageyama, T., Tanabe, K. and Koiwai, O. 1991. Development-dependent expression of isozymogens of monkey pepsinogens and structural differences between them. Eur. J. Biochem. 202: 205-215.

Kageyama, T. 2000. New world monkey pepsinogens A and C, and prochymosins. Purification, characterization of enzymatic properties, cDNA cloning, and molecular evolution. J. Biochem. 127: 761-770.

Kageyama, T. 2002. Pepsinogens, progastricsins, and prochymosins: Structure, function, evolution, and development. Cell. Mol. Life Sci. 59: 288-306.

Karlsen, S., Hough, E. and Olsen, R.L. 1998. Structure and proposed amino-acid sequence of a pepsin from Atlantic cod (*Gadus morhua*). Acta Crystallogr. D. 54: 32-46.

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.

Kim, J.S. and Park, J.W. 2004. Characterization of acid-soluble collagen from Pacific whiting surimi processing byproducts. J. Food Sci. 69: 637-642.

Kimura, S. and Ohno, Y. 1987. Fish type I collagen: tissue-specific existence of two molecular forms, $(\alpha1)_2\alpha2$ and $\alpha1\alpha2\alpha3$, in Alaska pollack. Comp. Biochem. Physiol. B. 88: 409-413.

Kimura, S., Ohno, Y., Miyauchi, Y. and Uchida, N. 1987. Fish skin type I collagen: wide distribution of an α3 subunit in teleosts. Comp. Biochem. Physiol. B. 88: 27-34.

Kimura, S. 1992. Wide distribution of the skin type I collagen α3 chain in bony fish. Comp. Biochem. Physiol. B. 102: 255-260.

Kishimura, H., Hayashi, K., Miyashita, Y. and Nonami, Y. 2006. Characteristics of trypsins from the viscera of true sardine (*Sardinops melanostictus*) and the pyloric ceca of arabesque greenling (*Pleurogrammus azonus*). Food Chem. 97: 65-70.

Kittiphattanabawon, P., Benjakul, S., Visessanguan, W., Nagai, T. and Tanaka, M. 2005. Characterisation of acid-soluble collagen from skin and bone of bigeye snapper (*Priacanthus tayenus*). Food Chem. 89: 363-372.

Kittiphattanabawon, P., Benjakul, S., Visessanguan, W., Kishimura, H. and Shahidi, F. 2010a. Isolation and characterisation of collagen from the skin of brownbanded bamboo shark (*Chiloscyllium punctatum*). Food Chem. 119: 1519-1526.

Kittiphattanabawon, P., Benjakul, S., Visessanguan, W. and Shahidi, F. 2010b. Isolation and properties of acid- and pepsin-soluble collagen from the skin of blacktip shark (*Carcharhinus limbatus*). Eur. Food Res. Technol. 230: 475-483.

Klomklao, S., Benjakul, S. and Visessanguan, W. 2004. Comparative studies on proteolytic activity of spleenic extract from three tuna species commonly used in Thailand. J. Food Biochem. 28: 355-372.

Klomklao, S., Benjakul, S., Visessanguan, W., Simpson, B.K. and Kishimura, H. 2005. Partitioning and recovery of proteinase from tuna spleen by aqueous two-phase systems. Process Biochem. 40: 3061-3067.

Klomklao, S., Kishimura, H., Yabe, M. and Benjakul, S. 2007. Purification and characterization of two pepsins from the stomach of pectoral rattail (*Coryphaenoides pectoralis*). Comp. Biochem. Physiol. B. 147: 682-689.

Klompong, V., Benjakul, S., Kantachote, D. and Shahidi, F. 2007. Antioxidative activity and functional properties of protein hydrolysate of yellow stripe trevally (*Selaroides leptolepis*) as influenced by the degree of hydrolysis and enzyme type. Food Chem. 102: 1317-1327.

Klompong, V., Benjakul, S., Yachai, M., Visessanguan, W., Shahidi, F. and Hayes, K.D. 2009. Amino acid composition and antioxidative peptides from protein hydrolysates of yellow stripe trevally (*Selaroides leptolepis*). J. Food Sci. 74: C126-C133.

Komsa-Penkova, R., Koynova, R., Kostov, G. and Tenchov, B. 2000. Discrete reduction of type I collagen thermal stability upon oxidation. Biophys. Chem. 83: 185-195.

Kristinsson, H.G. and Rasco, B.A. 2000. Fish protein hydrolysates: Production, biochemical, and functional properties. Crit. Rev. Food Sci. Nutr. 40: 43-81.

Kruger, N.J. 1996. The Bradford method for protein quantitation. *In* The protein protocols handbook. (Walker, J. M., ed.). p. 15-20. Humana Press Inc. New Jersey.

Kubota, M. and Ohnuma, A. 1970. Studies on bonito pepsin-II. Enzymic properties of bonito. Bulletin of the Japanese Society of Scientific Fisheries. 36: 1152-1156.

Kula, M.R. 1979. Extraction and purification of enzymes using aqueous two-phase systems. Appl. Biochem. Bioeng. 2: 71-95.

Laemmli, U.K. 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature. 227: 680-685.

Lawal, O.S. 2004. Functionality of African locust bean (*Parkia biglobossa*) protein isolate: effects of pH, ionic strength and various protein concentrations. Food Chem. 86: 345-355.

Lee, J.C. and Lee, L.L. 1981. Preferential solvent interactions between proteins and polyethylene glycols. J. Biol. Chem. 256: 625-631.

Lee, S.-H., Qian, Z.-J. and Kim, S.-K. 2010. A novel angiotensin I converting enzyme inhibitory peptide from tuna frame protein hydrolysate and its antihypertensive effect in spontaneously hypertensive rats. Food Chem. 118: 96-102.

Lin, L.H. and Chen, K.M. 2006. Preparation and surface activity of gelatin derivative surfactants. Colloids Surf. Physicochem. Eng. Aspects. 272: 8-14.

Lin, X.L., Wong, R.N.S. and Tang, J. 1989. Synthesis, purification, and active site mutagenesis of recombinant porcine pepsinogen. J. Biol. Chem. 264: 4482-4489.

Lineweaver, H. and Burk, D. 1934. The determination of enzyme dissociation constants. J. Am. Chem. Soc. 56: 658-666.

Liu, H., Li, D. and Guo, S. 2007. Studies on collagen from the skin of channel catfish (*Ictalurus punctaus*). Food Chem. 101: 621-625.

Liu, L.L. and Pigott, G.M. 1981. Preparation and use of inexpensive crude pepsin for enzyme hydrolysis of fish. J. Food Sci. 46: 1569-1572.

Lowry, O.H., Rosebrough, N.J., Farr, A.L. and Randall, R.J. 1951. Protein measurment with the folin phenol reagent. J. Biol. Chem. 193: 265-275.

Ma, H., Forssell, P., Partanen, R., Seppänen, R., Buchert, J. and Boer, H. 2009. Sodium caseinates with an altered isoelectric point as emulsifiers in oil/water systems. J. Agric. Food Chem. 57: 3800-3807.

Marciniszyn Jr., J., Hartsuck, J.A. and Tang, J. 1976. Mode of inhibition of acid proteases by pepstatin. J. Biol. Chem. 251: 7088-7094.

Mathialagan, N. and Hansen, T.R. 1996. Pepsin-inhibitory activity of the uterine serpins. Proc. Natl. Acad. Sci. USA. 93: 13653-13658.

Matsui, R., Ishida, M. and Kimura, S. 1991. Characterization of an α3 chain from the skin type I collagen of chum salmon (*Oncoorhynchus keta*). Comp. Biochem. Physiol. B. 99: 171-174.

Mechanic, G.L., Katz, E.P., Henmi, M., Noyes, C. and Yamauchi, M. 1987. Locus of a histidine-based, stable trifunctional, helix to helix collagen cross-link: stereospecific collagen structure of type I skin fibrils. Biochemistry. 26: 3500-3509.

Miller, N.J., Rice-Evans, C., Davies, M.J., Gopinathan, V. and Milner, A. 1993. A novel method for measuring antioxidant capacity and its application to monitoring the antioxidant status in premature neonates. Clin. Sci. 84: 407-412.

Mohd Nasir, N.F., Raha, M.G., Kadri, N.A., Sahidan, S.I., Rampado, M. and Azlan, C.A. 2006. The study of morphological structure, phase structure and molecular structure of collagen-PEO 600K blends for tissue engineering application. Am. J. Biochem. Biotechnol. 2: 175-179.

Moriarty, D.J.W. 1973. The physiology of digestion of blue-green algae in the cichlid fish, *Tilapia nilotica*. J. Zool. Lond. 171: 25-39.

Mutilangi, W.A.M., Panyam, D. and Kilara, A. 1996. Functional properties of hydrolysates from proteolysis of heat-denatured whey protein isolate. J. Food Sci. 61: 270-303.

Muyonga, J.H., Cole, C.G.B. and Duodu, K.G. 2004. Characterisation of acid soluble collagen from skins of young and adult Nile perch (*Lates niloticus*). Food Chem. 85: 81-89.

Nagai, T. and Suzuki, N. 2000. Isolation of collagen from fish waste material -- skin, bone and fins. Food Chem. 68: 277-281.

Nagai, T., Worawattanamateekul, W., Suzuki, N., Nakamura, T., Ito, T., Fujiki, K., Nakao, M. and Yano, T. 2000. Isolation and characterization of collagen from rhizostomous jellyfish (*Rhopilema asamushi*). Food Chem. 70: 205-208.

Nagai, T., Yamashita, E., Taniguchi, K., Kanamori, N. and Suzuki, N. 2001. Isolation and characterisation of collagen from the outer skin waste material of cuttlefish (*Sepia lycidas*). Food Chem. 72: 425-429.

Nagai, T., Araki, Y. and Suzuki, N. 2002. Collagen of the skin of ocellate puffer fish (*Takifugu rubripes*). Food Chem. 78: 173-177.

Nagai, T. and Suzuki, N. 2002. Preparation and partial characterization of collagen from paper nautilus (*Argonauta argo*, Linnaeus) outer skin. Food Chem. 76: 149-153.

Nagai, T., Suzuki, N. and Nagashima, T. 2008. Collagen from common minke whale (*Balaenoptera acutorostrata*) unesu. Food Chem. 111: 296-301.

Nalinanon, S., Benjakul, S., Visessanguan, W. and Kishimura, H. 2007. Use of pepsin for collagen extraction from the skin of bigeye snapper (*Priacanthus tayenus*). Food Chem. 104: 593-601.

Nalinanon, S., Benjakul, S., Visessanguan, W. and Kishimura, H. 2008a. Improvement of gelatin extraction from bigeye snapper skin using pepsin-aided process in combination with protease inhibitor. Food Hydrocolloid. 22: 615-622.

Nalinanon, S., Benjakul, S., Visessanguan, W. and Kishimura, H. 2008b. Tuna Pepsin: Characteristics and its use for collagen extraction from the skin of threadfin bream (*Nemipterus* spp.). J. Food Sci. 73: C413-C419.

Nalinanon, S., Benjakul, S., Visessanguan, W. and Kishimura, H. 2009. Partitioning of protease from stomach of albacore tuna (*Thunnus alalunga*) by aqueous two-phase systems. Process Biochem. 44: 471-476.

Nalinanon, S., Benjakul, S. and Kishimura, H. 2010a. Biochemical properties of pepsinogen and pepsin from the stomach of albacore tuna (*Thunnus alalunga*). Food Chem. 121: 49-55.

Nalinanon, S., Benjakul, S. and Kishimura, H. 2010b. Collagens from the skin of arabesque greenling (*Pleurogrammus azonus*) solubilized with the aid of acetic acid and pepsin from albacore tuna (*Thunnus alalunga*) stomach. J. Sci. Food Agric. In press.

Nalinanon, S., Benjakul, S. and Kishimura, H. 2010c. Purification and biochemical properties of pepsins from the stomach of skipjack tuna (*Katsuwonus pelamis*). Eur. Food Res. Technol. In press.

Narita, Y., Oda, S.I., Moriyama, A. and Kageyama, T. 2002. Primary structure, unique enzymatic properties, and molecular evolution of pepsinogen B and pepsin B. Arch. Biochem. Biophys. 404: 177-185.

Nguyen, A.-D., Nungaray, J., Martel, A., Le Goffic, F., Mollé, D. and Léonil, J. 1998. Purification and characterization of the main pepsinogen from the shark, *Centroscymnus coelolepis*. J. Biochem. 124: 287-293.

Nielsen, F.S. and Foltmann, B. 1993. Activation of porcine pepsinogen A. Eur. J. Biochem. 217: 137-142.

Nielsen, P.K. and Foltmann, B. 1995. Purification and characterization of porcine pepsinogen B and pepsin B. Arch. Biochem. Biophys. 322: 417-422.

Noda, M. and Murakami, K. 1981. Studies on proteinases from the digestive organs of sardine. II. Purification and characterization of two acid proteinases from the stomach. Biochim. Biophys. Acta. 658: 27-34.

Norris, E.R. and Mathies, J.C. 1953. Preparation, properties, and crystallization of tuna pepsin. J. Biol. Chem. 204: 673-680.

Novotná, L., Hrubý, M., Beneš, M.J. and Kučerová, Z. 2005. Affinity chromatography of porcine pepsin A using quinolin-8-ol as ligand. J. Chromatogr. A. 1084: 108-112.

Ogawa, M., Moody, M.W., Portier, R.J., Bell, J., Schenxnayder, M.A. and Losso, J.N. 2003. Biochemical properties of black drum and sheephead seabream skin collagen. J. Agric. Food Chem. 51: 8088-8092.

Oliveira, L.A., Sarubbo, L.A., Porto, A.L.F., Campos-Takaki, G.M. and Tambourgi, E.B. 2002. Partition of trypsin in aqueous two-phase systems of poly(ethylene glycol) and cashew-nut tree gum. Process Biochem. 38: 693-699.

Ong, E.B. and Perlmann, G.E. 1968. The amino-terminal sequence of porcine pepsinogen. J. Biol. Chem. 243: 6104-6109.

Owen, T.G. and Wiggs, A.J. 1971. Thermal compensation in the stomach of the brook trout (*Salvelinus fontinalis* Mitchill). Comp. Biochem. Physiol. B. 40: 465-473.

Pavlisko, A., Rial, A., De Vecchi, S. and Coppes, Z. 1997. Properties of pepsin and trypsin isolated from the digestive tract of *Parona signata* "Palometa". J. Food Biochem. 21: 289-308.

Pavlisko, A., Vecchi, S.D. and Coppes, Z. 1999. Enzymatic digestion of muscle from menhaden (*Brevoortia* spp.) at different pHs. J. Food Biochem. 23: 451-467.

Payne, K.J. and Veis, A. 1988. Fourier transform IR spectroscopy of collagen and gelatin solutions: deconvolution of the amide I band for conformational studies. Biopolymers. 27: 1749-1760.

Pearce, K.N. and Kinsella, J.E. 1978. Emulsifying properties of proteins: evaluation of a turbidimetric technique. J. Agric. Food Chem. 26: 716-723.

Pérez-Mateos, M. and Lanier, T.C. 2006. Comparison of Atlantic menhaden gels from surimi processed by acid or alkaline solubilization. Food Chem. 101: 1223-1229.

Petibois, C., Gouspillou, G., Wehbe, K., Delage, J.-P. and Déléris, G. 2006. Analysis of type I and IV collagens by FT-IR spectroscopy and imaging for a molecular investigation of skeletal muscle connective tissue. Anal. Bioanal. Chem. 386: 1961-1966.

Phillips, L.G., Whitehead, D.M. and Kinsella, J.E. 1994. Protein stabilized foams. *In* Structure-function of food proteins. (Phillips, L. G. *et al.*, eds.). p. 131–152. Academic Press. New York.

Pichová, I. and Kostka, V. 1990. Molecular characteristics of pepsinogen and pepsin from duck grandular stomach. Comp. Biochem. Physiol. B. 97: 89-94.

Piez, K.A. and Gross, J. 1960. The amino acid composition of some fish collagens: the relation between composition and structure. J. Biol. Chem. 235: 995-998.

Pillai, S. and Zull, J.E. 1985. Effects of ATP, vanadate, and molybdate on cathepsin D-catalyzed proteolysis. J. Biol. Chem. 260: 8384-8389.

Plepis, A.M.D.G., Goissis, G. and Das-Gupta, D.K. 1996. Dielectric and pyroelectric characterization of anionic and native collagen. Polym. Eng. Sci. 36: 2932-2938.

Raa, J. 1990. Biotechnology in aquaculture and the fish processing industry. A success story in Norway. *In* Advances in fisheries technology and biotechnology for increased profitability. (Voigt, M. N. and Botta, J. R., eds.). p. 509-524. Technomic. Lancester.

Rajagopalan, T.G., Stein, W.H. and Moore, S. 1966. The inactivation of pepsin by diazoacetylnorleucine methyl ester. J. Biol. Chem. 241: 4295-4297.

Regenstein, J.M. and Zhou, P. 2007. Collagen and gelatin from marine by-products. *In* Mazimising the Value of Marine By-Products. 1st Ed. (Shahidi, F., ed.). p. 279-303. Woodhead Publishing Limited. Cambridge.

Reh, G., Nerli, B. and Picó, G. 2002. Isolation of alpha-1-antitrypsin from human plasma by partitioning in aqueous biphasic systems of polyethyleneglycol-phosphate. J. Chromatogr. B. 780: 389-396.

Reh, G., Spelzini, D., Tubío, G., Picó, G. and Farruggia, B. 2007. Partition features and renaturation enhancement of chymosin in aqueous two-phase systems. J. Chromatogr. B. 860: 98-105.

Rich, D.H. and Sun, E.T.O. 1980. Mechanism of inhibition of pepsin by pepstatin. Effect of inhibitor structure on dissociation constant and time-dependent inhibition. Biochem. Pharmacol. 29: 2205-2212.

Richter, C., Tanaka, T. and Yada, R.Y. 1998. Mechanism of activation of the gastric aspartic proteinases: pepsinogen, progastricsin and prochymosin. Biochem. J. 335: 481-490.

Rigby, B.J. 1968. Amino-acid composition and thermal stability of the skin collagen of the antarctic ice-fish. Nature. 219: 166-167.

Rito-Palomares, M. 2004. Practical application of aqueous two-phase partition to process development for the recovery of biological products. J. Chromatogr. B. 807: 3-11.

Rochdi, A., Foucat, L. and Renou, J.-P. 2000. NMR and DSC studies during thermal denaturation of collagen. Food Chem. 69: 295-299.

Ruiz-Giménez, P., Burguete, M.C., Castelló-Ruiz, M., Marcos, J.F., Salom, J.B., Vallés, S., Torregrosa, G., Alborch, E. and Manzanares, P. 2007. Bovine lactoferrin pepsin hydrolysate exerts inhibitory effect on angiotensin I-converting enzyme-dependent vasoconstriction. Int. Dairy J. 17: 1212-1215.

Sadowska, M., Kołodziejska, I. and Niecikowska, C. 2003. Isolation of collagen from the skins of Baltic cod (*Gadus morhua*). Food Chem. 81: 257-262.

Saito, M., Kunisaki, N., Urano, N. and Kimura, S. 2002. Collagen as the major edible component of sea cucumber (*Stichopus japonicus*). J. Food Sci. 67: 1319-1322.

Sánchez-Chiang, L., Cisternas, E. and Ponce, O. 1987. Partial purification of pepsins from adult and juvenile salmon fish *Oncorhynchus keta*. Effect of NaCl on proteolytic activities. Comp. Biochem. Physiol. B. 87: 793-797.

Saravanan, S., Rao, J.R., Murugesan, T., Nair, B.U. and Ramasami, T. 2007. Partition of tannery wastewater proteins in aqueous two-phase poly (ethylene glycol)-magnesium sulfate systems: Effects of molecular weights and pH. Chem. Eng. Sci. 62: 969-978.

Shahidi, F., Han, X.-Q. and Synowiecki, J. 1995. Production and characteristics of protein hydrolysates from capelin (*Mallotus villosus*). Food Chem. 53: 285-293.

Shamsuzzaman, K. and Haard, N.F. 1985. Milk clotting and cheese making properties of a chymosin-like enzyme from harp seal mucosa. J. Food Biochem. 9: 173-192.

Shimada, K., Fujikawa, K., Yahara, K. and Nakamura, T. 1992. Antioxidative properties of xanthan on the antioxidation of soy bean oil in cyclodextrin emulsion. J. Agric. Food Chem. 40: 945–948.

Sielecki, A.R., Fujinaga, M., Read, R.J. and James, M.N.G. 1991. Refined structure of porcine pepsinogen at 1.8 Å resolution. J. Mol. Biol. 219: 671-692.

Sikorski, Z.E. and Naczk, M. 1981. Modification of technological properties of fish protein concentrates. Crit. Rev. Food Sci. Nutr. 14: 201-230.

Sikorski, Z.E., Scott, D.N. and Buisson, D.H. 1984. The role of collagen in the quality and processing of fish. Crit. Rev. Food Sci. Nutr. 20: 301-343.

Simpson, B.K. and Haard, N.F. 1984. Trypsin from greenland cod, *Gadus ogac*. Isolation and comparative properties. Comp. Biochem. Physiol. B. 79: 613-622.

Spelzini, D., Farruggia, B. and Picó, G. 2005. Features of the acid protease partition in aqueous two-phase systems of polyethylene glycol-phosphate: Chymosin and pepsin. J. Chromatogr. B. 821: 60-66.

Spelzini, D., Picó, G. and Farruggia, B. 2006. Dependence of chymosin and pepsin partition coefficient with phase volume and polymer pausidispersity in polyethyleneglycol-phosphate aqueous two-phase system. Colloids Surf., B. 51: 80-85.

Spelzini, D., Peleteiro, J., Picó, G. and Farruggia, B. 2008. Polyethyleneglycol-pepsin interaction and its relationship with protein partitioning in aqueous two-phase systems. Colloids Surf., B. 67: 151-156.

Squires, E.J., Haard, N.F. and Feltham, L.A.W. 1986. Gastric proteases of the Greenland cod *Gadus ogac*. I. Isolation and kinetic properties. Biochemistry and Cell Biology. 64: 205-214.

Srinivas, N.D., Barhate, R.S. and Raghavarao, K.S.M.S. 2002. Aqueous two-phase extraction in combination with ultrafiltration for downstream processing of Ipomoea peroxidase. J. Food Eng. 54: 1-6.

Steel, R.D. and Torrie, J.H. 1980. Principle and procedures of statistic: A biomaterial approach. 2nd Ed. McGraw-Hill. New York.

Streicher, E., Naudé, R.J. and Oelofsen, W. 1985. The isolation and characterization of pepsinogens from the proventriculus of the ostrich *Struthio camelus*. Comp. Biochem. Physiol. B. 82: 67-72.

Subasinghe, S. 1996. Innovative and value-added tuna products and markets. INFOFISH Intern. 1: 43-50.

Surówka, K. and Fik, M. 1994. Studied on the recovery of proteinaceous substances from chicken heads: II—Application of pepsin to the production of protein hydrolysate. J. Sci. Food Agric. 65: 289-296.

Suzuki, M., Narita, Y., Oda, S.I., Moriyama, A., Takenaka, O. and Kageyama, T. 1999. Purification and characterization of goat pepsinogens and pepsins. Comp. Biochem. Physiol. B. 122: 453-460.

Tang, J. 1971. Specific and irreversible inactivation of pepsin by substrate-like epoxides. J. Biol. Chem. 246: 4510-4517.

Tanji, M., Kageyama, T. and Takahashi, K. 1988. Tuna pepsinogens and pepsins. Purification, characterization and amino-terminal sequences. Eur. J. Biochem. 177: 251-259.

Tanji, M., Yakabe, E., Kageyama, T. and Takahashi, K. 1996. The primary structure of the major pepsinogen from the gastric mucosa of tuna stomach. J. Biochem. 120: 647-656.

Tanji, M., Yakabe, E., Kageyama, T., Yokobori, S.-i., Ichinose, M., Miki, K., Ito, H. and Takahashi, K. 2007. Purification and characterization of pepsinogens from the gastric mucosa of African coelacanth, *Latimeria chalumnae*, and properties of the major pepsins. Comp. Biochem. Physiol. B. 146: 412-420.

Tanji, M., Yakabe, E., Kubota, K., Kageyama, T., Ichinose, M., Miki, K., Ito, H. and Takahashi, K. 2009. Structural and phylogenetic comparison of three pepsinogens from Pacific bluefin tuna: Molecular evolution of fish pepsinogens. Comp. Biochem. Physiol. B. 152: 9-19.

Tanuja, S., Srinivas, N.D., Raghava Rao, K.S.M.S. and Gowthaman, M.K. 1997. Aqueous two-phase extraction for downstream processing of amyloglucosidase. Process Biochem. 32: 635-641.

The Customs Department. 2007. Import statistics of canned tuna 2006 (online). Available: www.customs.go.th. (4 Jul 2007)

Tello-Solís, S.R. and Romero-García, B. 2001. Thermal denaturation of porcine pepsin: a study by circular dichroism. Int. J. Biol. Macromol. 28: 129-133.

Thiansilakul, Y., Benjakul, S. and Shahidi, F. 2007a. Compositions, functional properties and antioxidative activity of protein hydrolysates prepared from round scad (*Decapterus maruadsi*). Food Chem. 103: 1385-1394.

Thiansilakul, Y., Benjakul, S. and Shahidi, F. 2007b. Antioxidative activity of protein hydrolysate from round scad muscle using alcalase and flavourzyme. J. Food Biochem. 31: 266-287.

Tomita, M., Bellamy, W., Takase, M., Yamauchi, K., Wakabayashi, H. and Kawase, K. 1991. Potent antibacterial peptides generated by pepsin digestion of bovine lactoferrin. J. Dairy Sci. 74: 4137-4142.

Trelstad, R.L., Catanese, V.M. and Rubin, D.F. 1976. Collagen fractionation: separation of native types I, II and III by differential precipitation. Anal. Biochem. 71: 114-118.

Tubío, G., Nerli, B. and Picó, G. 2007. Partitioning features of bovine trypsin and α-chymotrypsin in polyethyleneglycol-sodium citrate aqueous two-phase systems. J. Chromatogr. B. 852: 244-249.

Twining, S.S., Alexander, P.A., Huibregtse, K. and Glick, D.M. 1983. A pepsinogen from rainbow trout. Comp. Biochem. Physiol. B. 75: 109-112.

Umezawa, H. 1976. Structures and activities of protease inhibitors of microbial origin. Methods Enzymol. 45: 678-695.

Vercaigne-Marko, D., Kosciarz, E., Nedjar-Arroume, N. and Guillochon, D. 2000. Improvement of *Staphylococcus aureus*-V8-protease hydrolysis of bovine haemoglobin by its adsorption on to a solid phase in the presence of SDS: Peptide mapping and obtention of two haemopoietic peptides. Biotechnol. Appl. Biochem. 31: 127-134.

Walsh, G. 2001. Protein purification and characterization. *In* Proteins: biochemistry and biotechnology. (Walsh, G., ed.). p. 103-106. John Willey & Sons, Ltd. Chichester.

Wang, J., Hu, J., Cui, J., Bai, X., Du, Y., Miyaguchi, Y. and Lin, B. 2008. Purification and identification of a ACE inhibitory peptide from oyster proteins hydrolysate and the antihypertensive effect of hydrolysate in spontaneously hypertensive rats. Food Chem. 111: 302-308.

Wang, L., An, X., Xin, Z., Zhao, L. and Hu, Q. 2007. Isolation and characterization of collagen from the skin of deep-sea redfish (*Sebastes mentella*). J. Food Sci. 72: E450-E455.

Whitaker, J.R. 1994. Principles of enzymology for the food sciences. 2nd Ed. Marcel Dekker. New York.

Wong, D.W.S. 1989. Mechanism and Theory in Food Chemistry. Van Nostrand Reinhold. New York.

Woo, J.-W., Yu, S.-J., Cho, S.-M., Lee, Y.-B. and Kim, S.-B. 2008. Extraction optimization and properties of collagen from yellowfin tuna (*Thunnus albacares*) dorsal skin. Food Hydrocolloid. 22: 879-887.

Wu, T., Sun, L.-C., Du, C.-H., Cai, Q.-F., Zhang, Q.-B., Su, W.-J. and Cao, M.-J. 2009a. Identification of pepsinogens and pepsins from the stomach of European eel (Anguilla anguilla). Food Chem. 115: 137-142.

Wu, G.C.-C., Chiang, H.-C., Chen, K.-S., Hsu, C.-C. and Yang, H.-Y. 2009b. Population structure of albacore (*Thunnus alalunga*) in the Northwestern Pacific Ocean inferred from mitochondrial DNA. Fish. Res. 95: 125-131.

Wu, H.-C., Chen, H.-M. and Shiau, C.-Y. 2003. Free amino acids and peptides as related to antioxidant properties in protein hydrolysates of mackerel (*Scomber austriasicus*). Food Res. Int. 36: 949-957.

Xia, J., Dubin, P.L. and Kokufuta, E. 1993. Dynamic and electrophoretic light scattering of a water-soluble complex formed between pepsin and polyethylene glycol. Macromolecules. 26: 6688-6690.

Xu, R.A., Wong, R.J., Rogers, M.L. and Fletcher, G.C. 1996. Purification and characterization of acidic proteases from the stomach of the deep water finfish orange roughy (*Hoplostethus atlanticus*). J. Food Biochem. 20: 31-48.

Yakabe, E., Tanji, M., Ichinose, M., Goto, S., Miki, K., Kurokawa, K., Ito, H., Kageyama, T. and Takahashi, K. 1991. Purification, characterization, and amino acid sequences of pepsinogens and pepsins from the esophageal mucosa of bullfrog (*Rana catesbeiana*). J. Biol. Chem. 266: 22436-22443.

Yan, M., Li, B., Zhao, X., Ren, G., Zhuang, Y., Hou, H., Zhang, X., Chen, L. and Fan, Y. 2008. Characterization of acid-soluble collagen from the skin of walleye pollock (*Theragra chalcogramma*). Food Chem. 107: 1581-1586.

Yang, S., Huang, Z., Jiang, Z. and Li, L. 2008. Partition and purification of a thermostable xylanase produced by Paecilomyces thermophila in solid-state fermentation using aqueous two-phase systems. Process Biochem. 43: 56-61.

Yata, M., Yoshida, C., Fujisawa, S., Mizuta, S. and Yoshinaka, R. 2001. Identification and characterization of molecular species of collagen in fish skin. J. Food Sci. 66: 247-251.

You, L., Zhao, M., Regenstein, J.M. and Ren, J. 2010. Changes in the antioxidant activity of loach (*Misgurnus anguillicaudatus*) protein hydrolysates during a simulated gastrointestinal digestion. Food Chem. 120: 810-816.

Yu, Y., Hu, J., Bai, X., Du, Y. and Lin, B. 2006. Preparation and function of oligopeptide-enriched hydrolysate from globin by pepsin. Process Biochem. 41: 1589-1593.

Zaslavsky, B.Y. 1994. Partitioning of solutes in aqueous two-phase systems. *In* Aqueous two-phase partitioning: physical chemistry and bioanalytical applications. (Zaslavsky, B. Y., ed.). p. 153-220. Marcel Dekker Inc. New York.

Zayas, J.F. 1997. Functionality of Proteins in Food. Springer. Berlin.

Żelechowska, E., Sadowska, M. and Turk, M. 2010. Isolation and some properties of collagen from the backbone of Baltic cod (*Gadus morhua*). Food Hydrocolloid. 24: 325-329.

Zeng, S.-k., Zhang, C.-h., Lin, H., Yang, P., Hong, P.-z. and Jiang, Z. 2009. Isolation and characterisation of acid-solubilised collagen from the skin of Nile tilapia (*Oreochromis niloticus*). Food Chem. 116: 879-883.

Zhang, Y., Liu, W., Li, G., Shi, B., Miao, Y. and Wu, X. 2007. Isolation and partial characterization of pepsin-soluble collagen from the skin of grass carp (*Ctenopharyngodon idella*). Food Chem. 103: 906-912.

Zhou, Q., Fu, X.-P., Zhang, L.-J., Su, W.-J. and Cao, M.-J. 2007. Purification and characterization of sea bream (*Sparus latus* Houttuyn) pepsinogens and pepsins. Food Chem. 103: 795-801.

Zhou, Q., Liu, G.-M., Huang, Y.-Y., Weng, L., Hara, K., Su, W.-J. and Cao, M.-J. 2008. Pepsinogens and pepsins from Mandarin fish (*Siniperca chuatsi*). J. Agric. Food Chem. 56: 5401-5406.

Zimmermann, B.K., Pikkarainen, J., Fietzek, P.P. and Kuhn, K. 1970. Cross-Linkages in Collagen. Demonstration of Three Different Intermolecular Bonds. Eur. J. Biochem. 16: 217-225.

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List of Publication and Proceedings

Publications

1. Nalinanon, S., Benjakul, S., Visessanguan, W. and Kishimura, H. 2007. Use of pepsin for collagen extraction from the skin of bigeye snapper (*Priacanthus tayenus*). Food Chem. 104: 593-601.
2. Nalinanon, S., Benjakul, S., Visessanguan, W. and Kishimura, H. 2008. Improvement of gelatin extraction from bigeye snapper skin using pepsin-aided process in combination with protease inhibitor. Food Hydrocolloid. 22: 615-622.
3. Nalinanon, S., Benjakul, S., Visessanguan, W. and Kishimura, H. 2008. Tuna pepsin: Characteristics and its use for collagen extraction from the skin of threadfin bream (*Nemipterus spp.*). J. Food Sci. 73: C413-C419.
4. Nalinanon, S., Benjakul, S., Visessanguan, W. and Kishimura, H. 2009. Partitioning of protease from stomach of albacore tuna (*Thunnus alalunga*) by aqueous two-phase systems. Process Biochem. 44: 471-476.

5. Nalinanon, S., Benjakul, S. and Kishimura, H. 2010. Biochemical properties of pepsinogen and pepsin from the stomach of albacore tuna (*Thunnus alalunga*). Food Chem. 121: 49-55.
6. Kishimura, H., Klomklao, S., Nalinanon, S., Benjakul, S., Chun, B.S. and Adachi, K. 2010. Comparative study on thermal stability of trypsin from the pyloric ceca of threadfin hakeling (*Laemonema longipes*). J. Food Biochem. 34: 50-65.
7. Chun, B.S., Kishimura, H., Kanzawa, H., Klomklao, S., Nalinanon, S., Benjakul, S. and Ando, S. 2010. Application of supercritical carbon dioxide for preparation of starfish phospholipase A_2. Process Biochem. 45: 689-693.
8. Nalinanon, S., Benjakul, S. and Kishimura, H. 2010. Purification and biochemical properties of pepsins from the stomach of skipjack tuna (*Katsuwonus pelamis*). Eur. Food Res. Technol. 231: 259-269.
9. Ahmad, M., Benjakul, S. and Nalinanon, S. 2010. Compositional and physicochemical characteristics of acid solubilized collagen extracted from the skin of unicorn leatherjacket (*Aluterus monoceros*). Food Hydrocolloid. 24: 588-594.
10. Kishimura, H., Nagai, Y., Fukumorita, K., Adachi, K., Chiba, S., Nakajima, S., Saeki, H., Klomklao, S., Nalinanon, S., Benjakul, S. and Chun, B.-S. 2010. Acid- and heat-stable trypsin inhibitory peptide from the viscera of Japanese common squid (*Todarodes pacificus*). J. Food Biochem. In Press.
11. Nalinanon, S., Benjakul, S. and Kishimura, H. 2010. Collagens from the skin of arabesque greenling (*Pleurogrammus azonus*) solubilized with the aid of acetic acid and pepsin from albacore tuna (*Thunnus alalunga*) stomach. J. Sci. Food Agric. In press.
12. Nalinanon, S., Benjakul, S. and Kishimura, H. 2010. Type I collagen from the skin of ornate threadfin bream (*Nemipterus hexodon*): Characteristics and effect of pepsin hydrolysis. Food Chem. Submitted.
13. Nalinanon, S., Benjakul, S. and Kishimura, H. 2010. Functionalities and antioxidative properties of protein hydrolysates from the muscle of ornate threadfin bream treated with pepsin from skipjack tuna (*Katsuwonus pelamis*). Food Chem. Submitted.

Proceedings

1. Trangwacharakul, S., Auchariyamet, S., Nalinanon, S., Maksutthipan, S., Arsa, N., Nuanyai, T., Sittisamang, D., Srikumlaithong, S. and Chanpongsri, S. 2004. Process development of concentrated fish soup in pilot plant. Financial analysis on production of concentrated fish soup. Class. Invest. No. 44-09, Report No. 6, TISTR., Bangkok. 118p.
2. Sassanarakkit, S., Trangwacharakul, S., Nalinanon, S., Gumarathi, R., Chanpongsri, S., Suntorn, N. and Chansong, R. 2005. Research and development of shrimp feed supplement from feed canning industrial waste in the pilot plant. Class. Invest. No. 44-09, Report No. 8, TISTR., Bangkok, 52p.
3. Nalinanon, S., Benjakul, S., Visessanguan, W. and Nagai, T. 2006. Characterization of fish pepsin and its use for gelatin extraction from bigeye snapper (*Priacanthus tayenus*) skin. The 8th Agro-Industrial Conference: Food Innovation. Bitec Bangna, Bangkok, Thailand. June 15th-16th. Poster Presentation.
4. Nalinanon, S., Benjakul, S. and Visessanguan, W. 2007. Characteristics and functional properties of gelatin from bigeye snapper skin extracted using pepsin-aided process. The 33rd Congress on Science and Technology of Thailand (STT. 33): Science and Technology for Global Sustainability. Walailak University, Nakon Si Thammarat, Thailand, 18th-20th October. Poster Presentation.
5. Nalinanon, S. and Benjakul, S. 2008. Extraction and characterization of collagen from the skin of unicorn leatherjacket (*Aluterus monoceros*) solubilized by albacore tuna pepsin. The 6th Regional IMT-GT UNINET Conference 2008: Sustaining Natural Resources Towards Enhancing the Quality of Life Within the IMT-GT Zone. Penang, Malaysia, 28th-30th August. Poster Presentation.
6. Nalinanon, S., Benjakul, S., Kihimura, H. and Saeki, H. 2009. Biochemical properties of pepsinogen and pepsin from the stomach of albacore tuna (*Thunnus alalunga*). The Japanese Society of Fisheries Science, Spring Meeting 2009. Tokyo University of Marine Science and Technology, Tokyo, Japan, 27th-31st March. Poster Presentation.