Characterization and Use of Proteinase from Pyloric Caeca of Bigeye Snapper (*Priacanthus macracanthus*) for Production of Gelatin Hydrolysate with Antioxidative Activity

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ชื่อวิทยานิพนธ์ การจำแนกลักษณะและการประยุกต์ใช้เอนไซม์โปรตีเนสจากไส้ติ่งปลาตาหวาน (*Priacanthus macracanthus*) สำหรับการผลิตเจลาตินไฮโดรไลเสตที่มีกิจกรรมต้านออกซิเดชัน

ผู้เขียน นางสาวพรรณนิภา พันธุรัตน์

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บทคัดย่อ

จากการเตรียมส่วนสกัดจากไส้ติ่งปลาตาหวานหนังบาง (PCE) ที่มีกิจกรรมของเอนไซม์โปรตีเอส โดยการตกตะกอนแยกส่วนด้วยเกลือแอมโมเนียมซัลเฟตอิ่มตัวร้อยละ 40-60 พบว่าได้ผลิตผลร้อยละ 43.8 และมีความบริสุทธิ์เพิ่มขึ้น 3.9 เท่า จากการศึกษาสภาวะที่เหมาะสมต่อการทำงานของ PCE พบว่า พีเอชและอุณหภูมิที่เหมาะสมต่อการย่อยสลายเคซีนและสารตั้งต้นสังเคราะห์ 2 ชนิด (BAPNA และ TAME) คือ 8.0 และ 55 องศาเซลเซียส ตามลำดับ กิจกรรมการย่อยสลายโปรตีนของ PCE ถูกยับยั้งด้วยสารยับยั้งเอนไซม์ทริปซินจากถั่วเหลือง (SBTI) ส่วน E-64 เปปสเตติน เอ และ EDTA มีผลยับยั้งเพียงเล็กน้อย เมื่อศึกษารูปแบบโปรตีนและกิจกรรมของเอนไซม์ด้วยเทคนิคอิเล็กโตรโฟรีซิส และ สับสเตรตเจลอิเล็กโตรโฟรีซิส พบว่าแถบโปรตีนที่มีกิจกรรมการย่อยสลายโปรตีนประกอบด้วย 2 แถบ ซึ่งมีขนาดน้ำหนักโมเลกุลเท่ากับ 24 และ 55 กิโลดาลตัน โดยแถบที่มีโมเลกุลเท่ากับ 24 กิโลดาลตันมีขนาดแถบใหญ่กว่า ดังนั้นเอนไซม์หลักใน PCE คือ ทริปซินหรือเอนไซม์ที่มีลักษณะใกล้เคียงกับทริปซิน

จากการศึกษากิจกรรมการเป็นสารต้านออกซิเดชันของเจลาตินไฮโดรไลเสตจากหนังปลาตาหวานหนังบาง ซึ่งผ่านการย่อยสลายด้วยเอนไซม์อัลคาเลส นิวเทรสและ PCE ที่ระดับการย่อยสลายต่างๆ พบว่าเมื่อระดับการย่อยสลายเพิ่มขึ้น กิจกรรมการต้านออกซิเดชันของเจลาตินไฮโดรไลเสต ซึ่งประกอบด้วย ประสิทธิภาพการจับอนุมูลอิสระ (DPPH และ ABTS) และความสามารถในการรีดิวซ์เฟอริก (FRAP) เพิ่มขึ้น ที่ระดับการย่อยสลายเดียวกัน (ร้อยละ 15) เจลาตินไฮโดรไลเสตที่ผ่านการย่อยสลายด้วยเอนไซม์นิวเทรส มีกิจกรรมในการจับอนุมูลอิสระ DPPH และความสามารถในการรีดิวซ์เฟอริกสูงสุด ขณะที่เจลาตินไฮโดรไลเสตที่ผ่านการย่อยสลายด้วย PCE มีกิจกรรมในการจับอนุมูลอิสระ ABTS สูงสุด เมื่อใช้เอมไซม์ 2 ชนิดร่วมกันในการย่อยสลายเจลาตินพบว่า การใช้เอนไซม์เอนไซม์อัลคาเลสผสมนิวเทรสสามารถผลิตเจลาตินไฮโดรไลเสตที่มีกิจกรรมในการจับอนุมูลอิสระ DPPH และความสามารถในการรีดิวซ์เฟอริกสูงสุด

ในขณะที่การใช้เอนไซม์อัลคาเลสและ PCE สามารถผลิตเจลาตินไฮโดรไลเสตที่มีกิจกรรมในการจับอนุมูลอิสระ ABTS สูงสุด เมื่อศึกษาการใช้เจลาตินไฮโดรไลเสตในการป้องกันปฏิกิริยาออกซิเดชันพบว่า เจลาตินไฮโดรไลเสตที่ผ่านการย่อยสลายด้วยเอนไซม์อัลคาเลสร่วมกับนิวเทรสหรือ PCE ที่ความเข้มข้น 500 และ 1000 ppm แสดงสมบัติการเป็นสารต้านออกซิเดชันในระบบกรดลิโนเลอิกและระบบเลซิทิน-ไลโพโซม เมื่อนำเจลาตินไฮโดรไลเสตทั้ง 2 ชนิดมาแยกด้วยเจลฟิลเตรชันโครมาโตกราฟีพบว่าเปปไทด์ที่มีกิจกรรมในการต้านอนุมูลอิสระ ABTS สูงสุดมีน้ำหนักโมเลกุลเท่ากับ 1.7 กิโลดาลตัน

จากการศึกษาสมบัติทางกายภาพ องค์ประกอบทางเคมี และสมบัติทางประสาทสัมผัสของน้ำแอปเปิ้ลเสริมเจลาตินไฮโดรไลเสตทั้ง 2 ชนิด ที่ระดับร้อยละ 0.1 และ 0.3 (น้ำหนักต่อปริมาตร) พบว่าน้ำแอปเปิ้ลเสริมเจลาตินไฮโดรไลเสตมีพีเอช สี และความเข้มของสีน้ำตาลเปลี่ยนแปลงเล็กน้อยแต่ไม่มีผลต่อค่าความเป็นกรด กิจกรรมการจับอนุมูลอิสระ DPPH และ ABTS ของน้ำแอปเปิ้ลเสริมเจลาตินไฮโดรไลเสตที่ได้จากการย่อยสลายด้วยเอนไซม์อัลคาเลสและ PCE ร้อยละ 0.3 เพิ่มขึ้นร้อยละ 24 และ 28 ตามลำดับ เมื่อทดสอบสมบัติทางประสาทสัมผัสไม่พบความแตกต่างของความชอบด้านสี ความขุ่น กลิ่น กลิ่นรส เนื้อสัมผัส และความชอบรวม ระหว่างน้ำแอปเปิ้ลและน้ำแอปเปิ้ลเสริมเจลาตินไฮโดรไลเสตที่ความเข้มข้นร้อยละ 0.1 และ 0.3 ดังนั้น เจลาตินไฮโดรไลเสตที่ผลิตโดยใช้โปรตีเนสทางการค้าและโปรตีเอสจากไส้ติ่งปลาตาหวานหนังบางสามารถใช้เป็นแหล่งของเปปไทด์ที่มีสมบัติต้านอนุมูลอิสระในน้ำผลไม้ โดยไม่มีผลเสียต่อคุณภาพโดยรวม

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ABSTRACT

Pyloric caeca extract (PCE) with proteolytic activity from bigeye snapper *(Priacanthus macracanthus)* pyloric caeca was prepared by fractionation using ammonium sulfate precipitation (40-60% saturation). Purification fold of 3.9 and yield of 43.8% were obtained. The highest proteolytic activity of PCE was observed at 55˚C and pH 8.0 when casein, N^{α}-Benzoyl-DL-arginine-*p*-nitroanilide (BAPNA) and N^{α}-*p*-Tosyl-L-arginine methyl ester hydrochloride (TAME) were used as the substrates. The activity of PCE was highly inhibited by 1 mg/ml soybean trypsin inhibitor (SBTI), whereas 0.1 mM E-64, 0.1 mM pepstatin A and 2 mM EDTA exhibited the negligible effect toward the activity. Two proteolytic activity bands with apparent molecular weights (MW) of 55 and 24 kDa were found when analyzed using sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and substrate-gel electrophoresis. Activity band with MW of 24 kDa was much larger than that of protein with MW of 55 kDa. The result suggested that trypsin or trypsin-like proteinase was most likely the major proteinase in PCE.

Antioxidative activity of gelatin hydrolysates from bigeye snapper skin with different degrees of hydrolysis (DH) prepared using Alcalase, Neutrase and PCE, was determined. Gelatin hydrolysate exhibited the increases in 2,2-diphenyl-1-picryl hydrazyl (DPPH), 2,2-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS) radical scavenging activity and ferric reducing antioxidative power (FRAP) as DH increased ($p<0.05$). At 15% DH, gelatin hydrolysate prepared using Neutrase showed the highest DPPH radical activity and FRAP ($p<0.05$), while, gelatin hydrolysate prepared using PCE exhibited the highest ABTS radical scavenging activity ($p<0.05$). Hydrolysate derived from gelatin using the combination of two enzymes with selected

DH was also prepared and tested for antioxidantive activity. Hydrolysate produced by the combination of Alcalase and Neutrase showed the highest DPPH radical scavenging activity and FRAP ($p<0.05$), whereas that prepared using Alcalase and PCE had the highest ABTS radical scavenging activity ($p<0.05$). Gelatin hydrolysate prepared using Alcalase in combination with Neutrase or PCE at 500 and 1,000 ppm exhibited the antioxidative activity in linoleic acid oxidation and lecithin liposome systems. Both gelatin hydrolysates were fractionated by Sephadex G-25 gel filtration chromatography. Antioxidative peptide of both gelatin hydrolysates with ABTS radical scavenging activity had the molecular mass of 1.7 kDa. The isolated peptide showed the high stability in temperature range of 80-100ºC and with pH range of 2-12.

Gelatin hydrolysates prepared using Alcalase in combination with PCE or Neutrase were added in apple juice at levels of 0.1 and 0.3% (w/v). Chemical composition, physical properties, antioxidative activity and sensory property were analyzed. Apple juice fortified with both gelatin hydrolysates had slight changes in color, pH and browning intensity. However, the addition of hydrolysate had no impact on total acidity. DPPH and ABTS radical scavenging activities of apple juice fortified with gelatin hydrolysate prepared using Alcalase and PCE at 0.3% increased by 24 and 28%, respectively. No differences in color, turbidity, odor, flavor, body and the overall likeness between apple juice and those fortified with 0.1 and 0.3% gelatin hydrolysates ($p>0.05$). Therefore, gelatin hydrolysate produced with commercial proteinase and proteinase from bigeye snapper pyloric caeca could be used as a source of antioxidative peptide in juice without detrimental effect on overall quality.

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CHAPTER 1

INTRODUCTION

Free radical-mediated lipid peroxidation, oxidative stress and antioxidants have been widely interested. Under normal conditions, reactive oxygen species (ROS) and free radicals are effectively eliminated by the antioxidant defense systems such as antioxidant enzymes and non-enzymatic factors (Qian et al., 2008). A shift in the balance between ROS generation and destruction to overproduction or decreased detoxification is associated with chronic diseases such as cardiovascular diseases, neurodegenerative disorders, diabetes and certain types of cancer (Ferreira et al., 2007). Lipid peroxidation that occurs in food products is responsible for the development of unacceptable flavor and taste, the decrease in shelf-life and the formation of potentially toxic reaction products (Pihlanto et al., 2008). Thus, antioxidants may function to prevent the formation of radicals or to detoxify free radicals. Artificial antioxidants such as BHT, BHA or n-propyl galllate exhibit strong antioxidant activity, but their use is strictly regulated because of potential health hazards (Giménez et al., 2008). Therefore, there is a growing interest to seek for antioxidant from natural sources including some dietary protein compounds. Bioactive peptides with antioxidative properties derived from various proteins by enzymatic hydrolysis have become a topic of great interest for pharmaceutical, health food and processing/preservation industries (Samaranayaka and Li-Chan, 2008).

Bigeye snapper (*Priacanthus macracanthus*) is one of the preferable species for surimi production in Thailand. During surimi processing, skin and viscera are generally produced and become by-products, which are commonly used as low-value animal feeds (Hau and Benjakul, 2006). Almost 30% of the fish waste generated during the filleting process consists of skin and bones with high collagen content (Nagai and Suzuki, 2000). Those materials can be used for the production of fish gelatin, which can provide an alternative source of food grade gelatin. Nevertheless, utilization of fish gelatin is limited due to its low gel strength and low gelling temperature, compared to those of mammals (Jongjareonrak et al., 2006). Therefore, the production of fish gelatin hydrolysate containing bioactive peptides that may act

as potential physiological modulators of metabolism can be an alternative of using fish gelatin (Kim et al., 2007). Gelatin hydrolysates from fish skin including Alaska pollack (Kim et al., 2001), jumbo squid (*Dosidicus gigas*showed) (Mendis et al., 2005) and cobia (*Rachycentron canadum*) (Yang et al., 2008) showed antioxidative activity via radical scavenging and retarded lipid peroxidation.

Fish viscera are generated during the processing constituting about 10–15% (depending on the species) of fish biomass (Bhaskar and Mahendrakar, 2008). They are potential sources of enzymes such as proteinases that may have some unique properties for industrial applications (Klomklao et al., 2005). Trypsin (EC 3.4.21.4) is one of the serine proteases in fish viscera and is commonly synthesized as a proenzyme by pancreatic acinar cells (Kishimura and Hayashi, 2002). Trypsin and trypsin-like enzymes have been isolated and characterized from intestine of Nile tilapia (Bezerra et al., 2001), pyloric caeca of Monterey sardine (Castillo-Yañez et al., 2005) and pyloric caeca of bluefish (Klomklao et al., 2007). Using proteases from fish viscera has an advantage over commercial proteases since enzyme costs can be greatly reduced (Shahidi et al., 1995). The use of enzymes to hydrolyze protein from processing waste into functional protein hydrolysates is an alternative to obtain value-added product with increased market value. The production of hydrolysate from fish skin gelatin with antioxidative activity can be a promising means to gain the high market value product and can to be implemented in fish processing or related industries.

Literature Review

1. Proteolytic enzyme

Protease is the generic name given to those enzymes hydrolyzing the peptide in proteins and some synthetic substrate and coded as the EC 3.4.11-99 (Klomklao, 2007). Proteases include peptidases and proteinases (García-Carreño and Hernández-Cortés, 2000). Proteases differ in their ability to hydrolyze various peptide bonds. Each type of protease has a specific kind of peptide bonds to be cleaved. Proteases from various sources differ greatly in their catalytic and physical properties (Han and Shahidi, 1995). Proteases can be classified based on their similarities to well characterized proteases, as trypsin-like, chymotrypsin-like, etc., their pH activity profiles as acid, neutral or alkaline proteases, substrate specificity and mechanism of catalysis (Haard and Simpson, 1994).

1.1 Fish digestive proteases

Fish viscera or internal organs are a relatively large portion of the animal round weight; approximately 5-17% (Gildberg, 1992; Aspmo et al., 2005; Kurtovic et al., 2006). Viscera, one of the most important by-products of fishing industry, are recognized as a potential source of digestive enzymes, especially proteases with high activity over a wide range of pH and temperature conditions (Cancre et al., 1999; Shahidi and Kamil, 2001). Those enzymes can be biotechnological tools in the food industry (Castillo-Yañez et al., 2004). Digestive proteases are hydrolytic in their action and catalyze the cleavage of peptide bonds with the participation of water molecules as reactants (Simpson, 2000). A variety of digestive proteolytic enzymes have been isolated from the internal organs of fish and the distribution of protease varies, depending on species and organs (Klomklao et al., 2004). The most important proteolytic enzymes from fish viscera are the aspartic protease, e.g. pepsin, and serine proteases, e.g. trypsin, chymotrypsin and elastase (Aspmo et al., 2005). Acidic proteases from fish stomachs display high activity between pH 2 and 4, while alkaline digestive proteases are most active between pH 8 and 10 (Simpson, 2000). Homologous digestive proteases from marine animals may also differ from one another in their response to specific inhibitors (Simpson, 2000). Those enzymes from

fish viscera can be isolated or purified, based on various differences in biological component extracts including solubility (precipitation with salts, solvents, etc.), size (dialysis, size exclusion chromatography), charge (ion exchange chromatography, electrophoresis), and affinity for specific ligands (affinity chromatography) (Janson and Rydén, 1998). Those proteases may have some unique properties for industrial applications, such as in the detergent, food, pharmaceutical, leather and silk industries (Haard, 1992; Kawai and Ikeda, 1972; Ooshiro, 1971). Fish digestive proteases could help the fisheries industry in this region to better clean fish, instead of using mechanical procedures, or to remove scales from fish skin (Haard and Simpson, 1994). Some proteases have been explored as food processing aids and as reducers of stick-water viscosity in fishmeal processing (An and Visessanguan, 2000).

1.2 Classification of digestive proteases from marine animals

According to the International Union of Applied Biochemists classification, proteases from fish and aquatic invertebrates may be classified into four major groups such as acidic and aspartic proteases, serine proteases, thiol or cysteine proteases and metalloproteases (Haard and Simpson, 1994).

1.2.1 Acid/Aspartyl proteases

Acid or aspartly proteases are a group of endoproteases characterized by high activity and stability at acid pH. This is the basis of their group name “acid” protease. They are referred to as “aspartyl” proteases (or carboxyl proteases) because their catalytic sites are composed of the carboxyl groups of two aspartic acid residues (Mihalyi, 1987). The primary structure of the zymogen includes a signal peptide (or presequence) and the so-called propart, whose autocatalytic cleavage leads to the formation of the active enzyme (Kageyama et al., 1989). The catalytic mechanism depends on the presence of two aspartic acid residues positioned roughly in the center of a deep cleft forming the active site and covered by a hairpin loop (flap) protruding from the N-terminal lobe of the molecule. Following propart cleavage, the flap is dislodged from the substrate cleft allowing enzyme–substrate interaction to occur (Carginale et al., 2004). Based on the EC system, all the acid/aspartyl proteases from marine animals have the first three digits in common: EC 3.4.23. The three common aspartyl proteases that have been isolated and characterized from the stomachs of

marine animals are pepsin, chymosin and gastricsin (Simpson, 2000). These enzymes are endopeptidases and active under acidic conditions (Gildberg, 1988).

Pepsin is assigned the number EC 3.4.23.1. It has preferential specificity for the aromatic amino acids phenylalanine, tyrosine and tryptophan. Pepsin has an extracellular function as the major gastric proteinase (Klomklao et al., 2007). Fish pepsin may also be present in many isoenzyme forms (Squires et al., 1986). Pepsins and pepsin-like enzymes have been isolated and characterized in several fish species such as Atlantic cod (Gildberg et al., 1990), Monterey sardine (Castillo-Yañez et al., 2004) and pectoral rattail (Klomklao et al., 2007). Most fish species contain two or three major pepsins with an optimum hemoglobin digestion at pH between 2 and 4 (Gildberg and Raa, 1983). Acidic pepsin-like enzymes were detected in stomach region of arowana fish at optimum pH of 1.5–2.0 (Natalia et al., 2004). Two pepsins (A and B) purified from the stomach of pectoral rattail (*Coryphaenoides pectoralis*) showed the maximal activity at pH 3.0 and 3.5, respectively, and had the same optimal temperature at 45°C using hemoglobin as a substrate (Klomklao et al., 2007).

1.2.2 Serine proteases

The serine proteases have been described as a group of endoproteases with a serine residue in their catalytic site. This family of proteinases is characterized by the presence of a serine residue, together with an imidazole group and an aspartyl carboxyl group in their catalytic sites. They are inhibited by diisopropylphospho fluoridate (DFP) through reaction with the hydroxyl group of the active site serine residue (Mihalyi, 1987). The proteases in serine subclass have the same first three digits: EC 3.4.21 (Simpson, 2000). Two types of serine proteinases have been recovered from fish pyloric caeca, namely trypsin and chymotrypsin (Han, 1993). Apart from these enzymes, collagenases, elastases and carboxypeptidases have also been recovered and identified from fish intestinal organs (Shahidi and Kamil, 2001). Serine proteases distributed in fish intestine possess high activity under alkaline rather than neutral pH (Walsh and Wilcox, 1970) and are generally similar to those of warm blooded animals with respect to their molecular size, amino acid composition and sensitivity to serine protease inhibitors (Haard and Simpson, 1994). Trypsin is assigned the code EC 3.4.21.4. Trypsin has a very narrow specificity for the peptide bonds on the carboxyl side of arginine and lysine (Simpson, 2000) and play major

roles in biological processes including digestion and activation of zymogens (Cao et al., 2000). Chymotrypsin is assigned a code of EC 3.4.21.1, and it has a much broader specificity than trypsin. It cleaves peptide bonds involving amino acids with bulky side chains and nonpolar amino acids such as tyrosine, phenylalanine, tryptophan and leucine. Elastase is designated as EC 3.4.21.11. Elastase exhibits preferential specificity for alanine, valine and glycine (Simpson, 2000). Trypsin and trypsin-like enzymes have been isolated and characterized from the viscera of a wide range of cold water and warm water fish, including the pyloric caeca of tambaqui (Bezerra et al., 2001), pyloric caeca of chinook salmon (Kurtovic et al., 2006), spleen of skipjack tuna (Klomklao et al., 2007), spleen of Monterey sardine (Castillo-Yañez et al., 2005) and intestine of Nile tilapia (Bezerra et al., 2005). The purified trypsin from the pyloric caeca of walleye pollock had the maximal activities at around pH 8.0 and 50ºC for the hydrolysis of N^{α}-*p*-tosyl-*L*-arginine methyl ester hydrochloride (TAME) (Kishimura et al., 2008). Hau and Benjakul (2006) reported that trypsin from the pyloric caeca of bigeye snapper showed an optimal activity at 55ºC and pH range of 8–11 for hydrolysis of N^{α}-Benzoyl-DL-arginine-ρ-nitroanilide (BAPNA).

1.2.3 Thiol/cysteine proteases

Thiol or cysteine proteases are a group of endoproteinases that have cysteine and histidine residues as the essential groups in their catalytic sites. These enzymes require the thiol (-SH) group furnished by the active site cysteine residue to be intact, hence the group names “thiol” or “cysteine” proteases. The thiol proteases are inhibited by heavy metal ions and their derivatives, as well as by alkylating agents and oxidizing agents (Mihalyi, 1987). The first three digits common to thiol proteinases are EC 3.4.22. An example of a thiol proteinase from the digestive glands of marine animals is cathepsin B, which is designated as EC 3.4.22.1 (Simpson, 2000). The cathepsins can be subdivided into more than 10 subfamilies on the basis of their primary sequence and substrate preferences (Barrett and Rawlings, 1996). Cathepsins from different species display maximum activity over a broad pH range, from 3.5 in some to 8.0 in others. However, not all cathepsins are thiol proteinases (Simpson, 2000). Cathepsin B from hepatopancreas (a digestive gland) of carp (*Cyprinus carpio*) had a temperature maximum of 45ºC (pH 6) (Aranishi et al., 1997). Cardenas-Lopez and Haard (2009) reported that cathepsin L from jumbo squid

(*Dosidicus gigas*) hepatopancreas had an optimum pH of 4.5 and optimum temperature of 55ºC.

1.2.4 Metalloproteases

Metalloproteases are hydrolytic enzymes whose activity depends on the presence of bound divalent cations. Chemical modification studies suggest that there may be at least one tyrosyl residue and one imidazole residue associated with the catalytic sites of metalloproteases (Whitaker, 1994). The metalloproteases are inhibited by chelating agent such as 1,10-phenanthroline, EDTA and sometimes by the simple process of dialysis. Most of the metalloproteases known are exopeptidases. They all have a common first three digit's EC 3.4.24 (Simpson, 2000). Sivakumar et al. (1999) reported that collagenolytic metalloprotease (gelatinase) from the hepatopancreas of the marine crab (*Scylla serrata*) with MW of 55 kDa had optimum temperature at 25°C and pH 7–7.5. It was completely inhibited by EDTA.

1.3 Fish trypsin

One of the main digestive proteases, which is detected in the pyloric caeca and intestine of fish, is trypsin (EC 3.4.21.4). Trypsin is a member of a large family of serine proteases and cleaves the peptide bond on the carboxyl side of arginine and lysine (Kishimura et al., 2007). It is synthesized in the pancreas and secreted as an inactive precursor, trypsinogen, from the pancreatic acinar cells together with chymotrypsin and proelastase (Rypniewski et al., 1994). Trypsin and trypsin-like enzyme have been isolated and identified in a wide array of cold water as well as warm water fish species (Shahidi and Kamil, 2001). Trypsin and trypsin-like proteolytic enzymes have been extracted, purified and characterized in several fish species including pancreatic tissue of carp (Cohen and Gertler, 1981), pyloric caeca of rainbow trout (Kristjansson, 1991), pyloric caeca of tambaqui (Bezerra et al., 2001), pyloric caeca of starfish (Kishimura and Hayashi, 2002), pyloric caeca of silk snapper (Rivera, 2003), intestine and pyloric caeca of white grunt (Munõz, 2004), intestine of Nile tilapia (Bezerra et al.,2005), viscera of Japanese anchovy (Kishimura et al., 2005), pyloric caeca of bigeye snapper (Hau and Benjakul, 2006), spleen of tongol tuna (Klomklao et al., 2006a), pyloric caeca of chinook salmon (Kurtovic et al., 2006), viscera of sardine (Bougatef et al., 2007), pyloric caeca of jacopever, elkhorn

sculpin (Kishimura et al., 2007), pyloric caeca of spotted goatfish (Souza et al., 2007), pyloric caeca of walleye Pollock (Kishimura et al., 2008), pyloric caeca of mandarin fish (Lu et al., 2008), hepatopancreas of cuttle fish (Balti et al., 2009). The characteristics of enzymes from marine invertebrates resemble those of mammalian and fish trypsins in molecular weight, cleavage specificities, pH stability and reaction with inhibitors (Balti et al., 2009). However, marine invertebrate trypsins were unstable at acidic pH and were not activated or stabilized by adding calcium ions, unlike mammalian pancreatic trypsin (Kishimura and Hayashi, 2002).

Generally, fish trypsin has been reported to have the molecular weight in the range of 23–28 kDa (Hau and Benjakul, 2006). Castillo-Yañez et al. (2004) found that the molecular weight of isolated trypsin from the pyloric caeca of Monterey sardine was 25 kDa. El-Beltagy et al. (2005) reported that purified alkaline protease from the viscera of Bolti fish had a molecular weight of 23 kDa. The molecular weights of trypsin purified from the pyloric caeca of arabesque greenling, skipjack tuna and walleye pollock were estimated to be 24 kDa (Kishimura et al., 2006a; Klomklao et al., 2007; Kishimura et al., 2008). Their pH optima for the hydrolysis of various substrates have been reported to range from 8 to 11, while their optimal temperature for hydrolysis of those substrates ranged from 35 to 70ºC (Table 1). Trypsins from marine animals tend to be more stable at alkaline pH, but are unstable at acid pH, unlike mammalian trypsins that are most stable at acidic pH (Simpson, 2000).

Table 1 Optimum pH and temperature of fish trypsins

Identified species	Optimum pH	Optimum temperature (ºC)	Substrates	References
Anchovy (*Engraulis japonica*)	9	45	Casein	Heu et al. (1995)
	8	45	BAPNA[a]	
Southwest Atlantic croaker (*Micropogonius fumieri*)	9.5	60	BAPA[b]	Pavisko et al. (1997)
Carp (*Cyprinus carpio*)	9	40 and 45	Boc-Phe-Ser-Arg-MCA[c]	Cao et al. (2000)
Starfish (*Asterina Pectinifera*)	8	55	TAME[d]	Kishimura and Hayashi (2002)
White grunt (*Haemulon plumierii*)	8	40	TAME	Munõz (2004)
Japanese anchovy (*Engraulis japonica*)	8	60	TAME	Kishimura et al. (2005)
Bigeye snapper (*Pricanthus macracanthus*)	8-11	55	BAPNA	Hau and Benjakul (2006)
Yellowfin tuna (*Thunnus albacores*)	8.5	55 and 65	TAME	Klomklao et al. (2006b)
Chinook salmon (*Oncorhynchus tshawytscha*)	8	60	BAPNA	Kurtovic et al. (2006)
Sardine (*Sardina pilchardus*)	8	60	BAEE[e]	Bougatef et al. (2007)
Bluefish (*Pomatomus saltatrix*)	9.5	55	BAPNA	Klomklao et al. (2007)
Skipjack tuna (*Katsuwonus pelamis*)	8.5	60	TAME	Klomklao et al. (2007)
Spotted goatfish (*Pseudupeneus maculatus*)	9	55	BAPNA	Souza et al. (2007)
Walleye pollock (*Theragra chalcogramma*)	8	50	TAME	Kishimura et al. (2008)
Mandarin fish (*Siniperca chuatsi*)	8.5	35 and 40	Boc-Phe-Ser-Arg-MCA	Lu et al. (2008)
Cuttlefish (*Sepia officinalis*)	8	70	BAPNA	Balti et al. (2009)

[a] N^{α}-Benzoyl-DL-arginine-*ρ*-nitroanilide.
[b] benzoyl-DL-arginine-*p*-nitroanilid·
[c] *t*-Butyloxy-carbonyl-Phe-Ser-Arg-4-methyl-coumaryl-7-amide.
[d] tosyl arginine methyl ester.
[e] N^{α}-benzoyl-L-arginine ethyl ester

2. Fish collagen

Collagen is abundant in tendons, skin, bone, the vascular system of animals and the connective tissue sheath surrounding muscle, contributing to toughness of muscle. About 10% of mammalian muscle protein is collagen but the amount in fish is generally much less. Some of collagen is soluble in neutral salt solution, some is soluble in acid and some is insoluble (Foegeding et al., 1996). The collagen monomer is a long cylindrical protein about 2,800 Å long and 14-15 Å in diameter (Foegeding et al., 1996). It consists of three polypeptide units (called α-chains). Each α-chain coils is a left-handed helix with three residues per turn, and three chains are twisted right-handed to form the triple helix held together by hydrogen bonding (Figure 1). Each α-chain contains ~1,000 amino acid residues and varies in amino acid compositions (Wong, 1989) and has a molecular mass of about 100,000 Da, yielding a total molecular mass of about 300,000 Da for collagen (Foegeding et al., 1996). Polypeptides of collagen are mostly helical but differ from the typical α-helix due to the abundance of hydroxyproline and proline, which interfere with α-helical structure (Foegeding et al., 1996). All collagens share certain characteristics, but differ mainly in the size of the molecule, the interruptions of the triple-helical domain and the formation of supramolecular structures (Kovanen, 1989). Various types of collagen are observed among different organs and connective tissue layers of muscular tissue (Table 2).

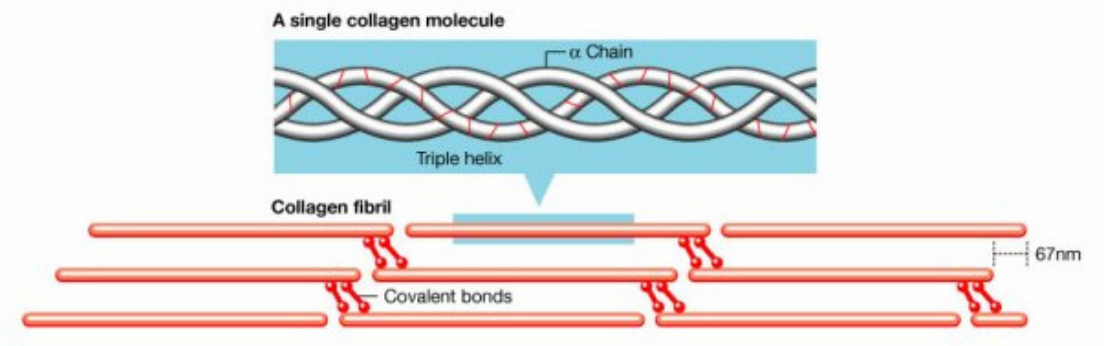

Figure 1 The structure of a typical collagen molecule.

Source : Mathews et al. (1999)

Table 2 Types of collagen

Type	Triple helix	Tissue distribution
I	Two identical α1(I) chains + one α2 chain	Skin, tendon, bone, muscle (epimysium), wall of blood vessel etc., except cartilage
II	Three α1(II) chains	Intervertebral disc, hyaline cartilage
III	Three α1(III) chains	Cardiovascular vessel, uterus, fetal skin, lung, inner organs, spleen, synovial membranes, muscle and aorta
IV	Three α1(IV) chains	Basement membrane, lung, kidney glomeruli, lens capsule and muscle (endomysium)

Source: Kovanen (1989), Wong (1989) and Burghagen (1999).

The prerequisite for the formation of homo- or heterotrimetric triple-helix is the repeating unique amino acid sequence $(\text{-Gly-X-Y-})_n$ in each α-chains to coil tightly around one another. The occurrence of proline and 4-hydroxyproline at X- and Y- positions, respectively, promotes the stability of the triple-helix by hydrogen bonding or by hydrogen water-bridges between the chains (Kovanen, 1989). Collagen contains high contents of glycine (33%) and proline (12%) with the occurrence of 4-hydroxyproline (12%) and 5-hydroxylysine (1%) (Burghagen, 1999). Glycine generally represents mainly one-third of the collagen molecule. The repetitive occurrence of glycine is absent in the first 14 amino acid residues from N-terminus and the first 10 from the C-terminus, with these end portions being termed "telopeptides" (Foegeding et al., 1996). Collagen is almost devoid of tryptophan (Jongjareonrak et al., 2006).

Collagen contents in fish muscles depend on the species as well as on the state of maturation and feeding of the fish (Regenstein and Regenstein, 1991). In starving fish, the sarcoplasmic and myofibrillar proteins undergo gradual degradation, while the connective tissues are not utilized. Collagen is deposited in the myocommata and in the skin. Generally, the contents of collagen in fish muscles range from about 1 to 12% of crude protein. Collagen at levels of 0.2-2.2% was found in fresh meat and 1.7-4.6% of collagen was reported in the fish skin (Sikorski et al., 1990). Fish muscle,

skin, bone and scale collagen differ from bovine and porcine hide collagens in having significantly higher contents of seven essential amino acids and a considerably lower concentration of hydroxyproline residues (Sikorski et al., 1990). Skin, bone, scales or fins collagen from several fish species have been isolated and characterized such as carp, cod, pike (Piez and Gross, 1960), hake (Montero et al., 1999), skipjack tuna, Japanese sea-bass, ayu, yellow sea bream, chub mackerel, bullhead shark, horse mackerel (Nagai and Suzuki, 2000), bigeye snapper (Kittiphattanabawon et al., 2005), brown backed toadfish (Senaratne et al., 2006), *Lates calcarifer* (Sankar et al., 2008) and deep-sea redfish (Wang et al., 2008). Collagen from bigeye snapper skin comprised two different α chains, α1 and α2 and were classified as type I collagen (Kittiphattanabawon et al., 2005). The denaturation temperature of fish collagens was lower than that of collagen from porcine skin (Nagai and Suzuki, 2000) because fish collagens contain lower imino acid contents than mammalian collagens (Foegeding et al., 1996). Moreover, the denaturation temperatures of collagens from cold-water fish were much lower compared to temperate and tropical fish species (Wang et al., 2008).

Collagen can be extracted from fish skin, bone and fin. Skin collagen was prepared from Japanese sea-bass, chub mackerel and bullhead shark with the yield of 51.4, 49.8 and 50.1%, respectively. Collagen was extracted from the bones of skipjack, Japanese sea-bass, ayu, yellow sea bream and horse mackerel with the yield of 42.3, 40.7, 53.6, 40.1 and 43.5%, respectively. Collagen was also extracted from the fin of from Japanese sea-bass with the yield of 5.2% (acid-soluble collagen) and 36.4% (acid-insoluble collagen) (Nagia and Suzuki, 2000). Jongjareonrak et al. (2006) isolated and characterized acid- and pepsin-solubilized collagen from bigeye snapper (*Priacanthus macracanthus*) skin with yields of 64 and 11 g/kg wet weight, respectively. The denaturation temperatures (T_{max}) of these collagens were as follows: skin collagen (25.0-26.5ºC), bone collagen (29.5-30.0ºC) and fin collagen (28.0-29.1ºC), respectively (Nagia and Suzuki, 2000). These values were about 7-12ºC lower than that of porcine skin collagen. Thermal transitions of acid- and pepsin-solubilized collagen from bigeye snapper were observed with T_{max} of 30.37 and 30.87 °C, respectively (Jongjareonrak et al., 2006).

3. Fish gelatin

Gelatin is derived from the thermal degradation of collagen. Gelatin can be extracted using two important steps: alkali treatment and hot-water extraction. The alkali treatment removes non-collagen protein of sample prior to hot-water extraction. Thermohydrolysis has been used to solubilize and separate the gelatin (Cho et al., 2004). Gelatin consists of random chains without triple helix. The process involves the disruption of non-covalent bonds and it is partially reversible during gelation process (Ward and Courts, 1977). During the collagen to gelatin transition, many non-covalent bonds are broken along with some covalent inter-and intramolecular bonds (Schiff base and aldo condensation bonds) and a few peptide bonds. This result is the conversion of helical collagen structure to a more amorphous form, known as "gelatin" (Figure 2). These changes constitute denaturation of the collagen molecule but not to the point of a completely unstructured product. If the latter happens, glue instead of gelatin is produced. After gelatin is produced and the temperature is lowered to below the critical value, there is a partial renaturation of the collagen molecule, involving what is called the "Collagen fold". Apparently, those parts of collagen that are rich in proline and hydroxyproline residues regain some of their structure, following which they can apparently interact (Foegeding et al., 1996).

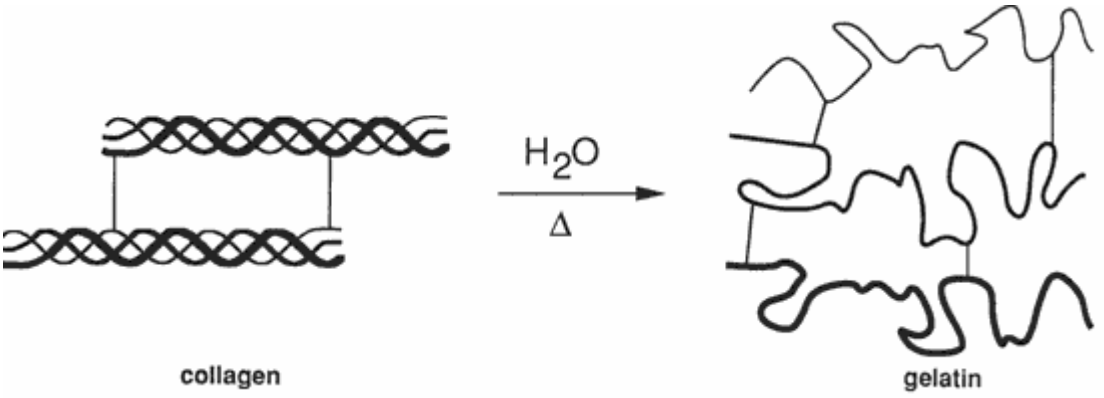

Figure 2 Denaturation of collagen.

Source : Hansen et al. (1991)

In general, there are two methods to obtain gelatin from skins and bones, an acid process (gelatin A with isoelectric point at pH 6–9) and an alkaline process (gelatin B with isoelectric points at pH 5) (Stainsby, 1987). Hydrochloric acid, sulfuric acid, phosphoric acid, calcium hydroxide and sodium hydroxide (Cho et al., 2005) are widely used for the manufacture of gelatin from mammals. However fish gelatin is easy to be hydrolyzed by acid or alkaline. Therefore, the pretreatment of fish skin must be mild. Calcium hydroxide, acetic acid and citric are generally used in the pretreatment of fish skin for its mild hydrolysis prior to gelatin extraction (Liu et al., 2009). The properties of gelatin, molecular weight, amino acid residues and number of polypeptide chains depend on the position of the breaks. Generally, the chemical composition of gelatin is similar to that of the parent collagen (Eastoe and Leach, 1977).

Gelatin from marine sources (fish skin, scales, bone and fins) has been looked upon as a possible alternative to bovine and porcine gelatin. Fish gelatin has been produced from black tilapia (*Oreochromis mossambicus*), red tilapia (*Oreochromis nilotica*) (Jamilah and Harvinder, 2002), Baltic cod (*Gadus morhua*) (Kołodziejsk et al., 2004), Nile perch (*Lates niloticus*) (Muyonga et al., 2004), yellowfin tuna (*Thunnus albacares*) (Cho et al., 2005), cod (*Gadus morhua*) (Arnesen and Gildberg, 2006), Atlantic salmon (*Salmo salar*) (Arnesen and Gildberg, 2007), sin croaker (*Johnius dussumieri*), shortfin scad (*Decapterus macrosoma*) (Cheow et al., 2007) and channel catfish (*Ictalurus Punctatus*) (Liu et al., 2009). Fish gelatin is generally produced by a mild acid treatment (type A gelatin) process (Gómez-Guillén and Montero, 2001). Gudmundsson and Hafsteinsson (1997) reported that the concentrations of sodium hydroxide, sulfuric and citric acids used for the extraction of gelatin from cod skins affected both yield and quality. The highest yield of gelatin (17%, w/w) was obtained when low concentrations (0.1–0.2%, w/v) of sulfuric acid and sodium hydroxide were applied to the skins, followed by treatment with 0.7% (w/v) citric acid. The gelatin yields have been reported to vary among the fish species, mainly due to the differences in collagen content, the compositions of skin as well as the skin matrix (Jongjareonrak et al., 2006). The different yields of skin gelatin have been reported for sole (8.3%), megrim (7.4%), cod (7.2%), and hake (6.5%) (Gómez-Guillén et al., 2002), young and adult Nile perch (12.5% and 16%, respectively)

(Muyonga et al., 2004), sin croaker (14.3%) and shortfin scad (7.25%) (Cheow et al., 2007). Jamilah and Harvinder (2002) extracted gelatin from the black and the red tilapias with yielding of 5.39 and 7.81%, respectively. Furthermore, the high degree of cross-linking via covalent bonds caused the decrease in solubility of collagen and might lead to the lower content of extractable gelatin (Foegeding et al., 1996). Gelatins from different fish species have different characteristics and properties. This different behaviors may be caused by the differences in the amino acid composition, the α1/α2 collagen-chain ratio, and the molecular weight distribution (Jongjareonrak et al., 2006). Gelatins from black and red tilapia had very high contents of glycine and threonine, and are essentially low in proline, while serine, tryptophan and histidine are not detectable (Jamilah and Harvinder, 2002). Generally, fish gelatin has lower gel strength and melting point than mammalian gelatin (Norland, 1987). Salmon gelatin expressed slightly higher gelling temperature (12ºC) than cod gelatin (10ºC) (Arnesen and Gildberg, 2007). Gómez-Guillén et al. (2002) reported that tropical-fish, such as tilapia, was a superior material for gelatin processing, compared with cold-water fish, which had poorer physical properties. The low hydroxyproline content in fish skin gelatin was a major reason for the low gel strength of these gelatins. It is well established that hydrogen bonds between water molecules and free hydroxyl groups of amino acids in gelatin are essential for gel strength (Arnesen and Gildberg, 2006). Gelatin with higher content of hydroxyproline is believed to have higher viscoelastic properties and its ability to develop triple helix structures, which are important for stabilizing the gelatin gel structure (Gómez-Guillén et al., 2002). Jongjareonrak et al. (2006) found that skin gelatins from bigeye snapper and brownstripe red snapper were rich in glycine, alanine and proline. The numbers of imino acids (proline and hydroxyproline) both species were higher than that reported in sole, megrim, cod and hake but lower than that found in porcine skin gelatin (Table 3). Arnesen and Gildberg (2007) reported that gelatin from salmon contained slightly more hydroxyproline and proline (16.6%) than cod gelatin (15.4%), whereas the content of serine was lower (4.6% versus 6.3%).

Table 3 Amino acid composition of fish gelatin as compared with two porcine skin gelatins (residues per 1000 total amino acid residues)

Amino acids	Source of skin gelatin						
	Bigeye snapper	Brownstripe red snapper	Sole	Megrim	Cod	Hake	Porcine
Hyp	91	84	61	60	50	59	108
Asx	61	56	48	48	52	49	51
Thr	32	31	20	20	25	22	18
Ser	38	39	44	41	64	49	35
Glx	103	105	72	72	78	74	83
Pro	134	141	113	115	106	114	114
Gly	193	204	352	350	344	331	329
Ala	103	108	122	123	96	119	115
Val	21	17	17	18	18	19	21
Met	17	15	10	13	17	15	9
Ile	10	9	8	8	11	9	12
Leu	27	25	21	21	22	23	24
Tyr	6	5	3	3	3	4	1
Phe	21	20	14	14	16	15	10
His	12	9	8	8	8	10	4
Hyl	-	-	5	5	6	5	-
Lys	38	38	27	27	29	28	29
Arg	92	94	55	54	56	54	38

Source: Gómez-Guillén et al. (2002), Cho et al. (2004) and Jongjareonrak et al. (2006)

4. Protein hydrolysates

Protein hydrolysates can be defined as proteins that are chemically or enzymatically broken down to peptides of varying sizes (Adler-Nissen, 1979). Biomedical, nutraceutical, antioxidative and functional properties including improved

solubility, emulsifying, foaming, gelation, water- and fat- holding capacities can be modified by hydrolysis process (Kim et al., 2001).

The enzymic digestion of proteins with different proteases is an efficient method of producing peptides with the improved nutritional properties and bioactivities (Korhonen and Pihlanto, 2003). Added enzymes are used to obtain a more selective hydrolysis since proteases are specific for peptide bonds adjacent amino acid residues (Peterson, 1978). Various physiological activities have been detected in the hydrolysates derived from the proteolytic hydrolysis of many food proteins, such as casein hydrolysate prepared using trypsin (Maruyama et al., 1985), hydrolysate from porcine skeleton muscle using trypsin, chymotrypsin, pronase E, proteinase K, thermolysin, ficin, papain and pepsin (Arihara et al., 2001), gelatin hydrolysate prepared using collagenase hydrolysate (Kim et al., 2001), hydrolysate from yellowfin frame protein prepared using chymotrypsin (Jung et al., 2006) and potato hydrolysate prepared using Alcalase, Neutrase and Esperase (Pihlanto et al., 2008). Several factors including pH, time, enzyme to substrate level and temperature, influence have the enzymatic activity and degree of hydrolysis (Liaset et al., 2000).

4.1 Type of enzyme and substrates

A wide variety of commercial enzymes has been used successfully to hydrolyze fish and other food proteins (Table 4). The choice of substrate, protease employed and the degree to which the protein is hydrolyzed generally affects the physicochemical properties of the resulting hydrolysates (Mullaly et al., 1995). Proteolytic enzymes from microorganisms such as Alcalase, Neutrase, Protease N and Protamex have been found to be more suitable to produce fish protein hydrolysate because of their high productivity (Benjakul and Morrisey, 1997). Enzyme from plants and animals such as papain, bromelain, ficin and pepsin are still used for hydrolysis (Liaset et al., 2000). Acid proteases, even though are better for microbial growth prevention, have only low protein yield. Thus, milder enzymes at neutral and slightly alkaline condition have been used more frequently (Kristinsson and Rasco, 2000). Due to high proteolytic activity of microbial proteases, those enzymes have been used intensively.

Table 4 Type of enzymes and substrates used to prepare protein hydrolysates

Enzymes	Substrates	References
Alcalase, Neutrase, Papain	Capelin (*Mallotus villosus*)	Shahidi et al. (1995)
Protamex	Atlantic salmon (*Salmo salar*, L.) frames	Liaset et al. (2002)
Thermolysin	Chum salmons (*Oncorhynchus keta*)	Ono et al. (2003)
Protease N	Mackerel (*Scomber austriasicus*)	Wu et al. (2003)
Alcalase, pancreatin	Shrimp (*Xiphopenaeus kroyeri*) waste	Holanda and Netto (2006)
Alcalase, Flavourzyme	Round scad (*Decapterus maruadsi*)	Thiansilakul et al. (2007)
Alcalase	Catla (*Catla catla*) viscera	Bhaskar et al. (2008)
Alcalase, Flavourzyme	Silver carp (*Hypophthalmichthys molitrix*)	Dong et al. (2008)
Papain	Grass carps (*Ctenopharyngodon idellus*)	Ren et al. (2008)
Trypsin	Hemp (*Cannabis sativa* L.)	Yin et al. (2008)
Papain, Protamex	Loach (*Misgurnus anguillicaudatus*)	You et al. (2008)
Protease N	Royal jelly	Guo et al. (2009)
Pepsin	Bovine casein	Miguel et al. (2009)

4.1.1 Alcalase

Alcalase (Subtilisin carlberg: EC 3.4.21.14) is an alkaline bacterial protease produced from *Bacillus licheniformis*. It has been proven to be one of the best enzyme used in the preparation of protein hydrolysate (Guerard et al., 2001). Alcalase is endopeptidase which is able to hydrolyze proteins with broad specificity for peptide bonds and prefers a large uncharged residue. Adler-Nissen (1986) reported that Alcalase was more active at alkaline pH and remained active to pH 6.0. The activity was high in pH range of 6.5-8.5 but showed considerable loss of activity at pH 10.5. Alcalase showed a high activity toward Pacific whiting waste in the high temperature range (55-70°C) with an optimum at 60°C (Benjakul and Morrissey, 1997). Normah et al. (2005) found that the hydrolysis of threadfin bream (*Nemipterus japonicus*) meat by Alcalase was optimum at 60ºC and pH 8.5 for 120 min using an enzyme substrate ratio of 2%. With these hydrolysis conditions, 20% hydrolysis was achieved and 70% nitrogen was recovered. Bhaskar et al. (2008) reported that the optimum conditions to obtain a higher degree of hydrolysis close to 50% using Alcalase for visceral wastes of fresh water carp were an enzyme to substrate level of 1.5% (v/w), pH 8.5, temperature of 50ºC and a hydrolysis time of 135 min. Fish protein hydrolysates prepared using Alcalase had less bitter taste as compared to those made with papain (Hoyle and Merritt, 1994). Alcalase has been documented to be a better candidate for hydrolyzing fish proteins based on enzyme cost per activity (Kristinsson and Rasco, 2000).

4.1.2 Neutrase

Neutrase is a neutral protease derived from *Bacillus amyloliquefaciens*, with a broad specificity. It exhibits the optimal activity at 45-55°C and pH 5.5-7.5. No activity is found at 80°C (Adler-Nissen, 1986). Benjakul and Morrissey (1997) found that Neutrase showed optimum activity against Pacific whiting solid wastes at pH 7.0 and 55 °C. Dilek and Belma (2007) reported that optimum conditions for hydrolysis and solubilization of corn gluten using Neutrase were 10 g/l protein concentration, 4 ml/l enzyme concentration, 45°C and pH 6.5. Jayaprakasha and Yoon (2005) found that spray dried whey protein concentrate modified by Neutrase had the decreased viscosity, improved solubility, increased foaming capacity and improved emulsifying.

4.2 Enzyme concentration

At increasing enzyme concentration upon the reaction rate, the substrate must be present in an excess amount. The reaction must be independent of the substrate concentration. Any change in the amount of product formed over a specified period of time will be dependent upon the level of enzyme present (Klompong et al., 2007). The relationship between activity and concentration is affected by many factors such as temperature, pH, etc. Degree of hydrolysis (DH) has been used as an indicator for the cleavage of peptide bond (Adler-Nissen, 1976). Cheftel et al. (1971) reported that an increase in enzyme concentration has a positive effect on overall proteolysis with subsequent increases in solubilization of fish protein concentrate. Benjakul and Morrissey (1997) found that when the enzyme concentration was increased, DH of Pacific whiting solid wastes treated with both Alcalase and Neutrase increased (Figure 3).

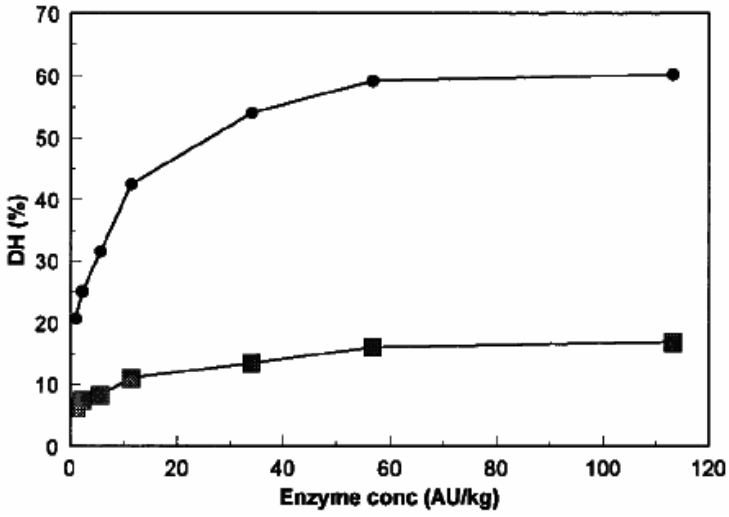

Figure 3 Effect of Alcalase and Neutrase concentration on DH of Pacific whiting solid wastes. Different amounts of enzyme were added to the suspension of PWSW in buffer (1:2 ratio, w/v). The reaction was run for 30 min at pH 9.5, 60°C and pH 7.0, 55°C for Alcalase and Neutrase, respectively.

Source: Benjakul and Morrissey (1997)

4.3 Hydrolysis time

The enzymatic hydrolysis of protein is characterized by an initial rapid phase, during which a large of peptide bonds are hydrolyzed. Thereafter, the rate of enzymatic hydrolysis decreases and reaches a stationary phase where no apparent hydrolysis takes place (Shahidi et al., 1995). Kim et al. (1990) reported a rapid rise in DH (about 65% of total) in the first 10 min of reaction when applying trypsin to soy protein isolates, a common characteristic in enzyme/protein systems and can be linked to saturation and/or inhibition phenomena in the substrate or product. Holanda and Netto (2006) reported that the hydrolysis curves of shrimp waste using Alcalase and pancreatin showed high initial reaction rates, followed by decreases in the reaction rate up to the stationary phase. With increasing hydrolysis time, DH of haemoglobin hydrolyzed by Alcalase and Flavourzyme increased rapidly in 1 h and then decreased (Chang et al., 2007). Dong et al. (2008) reported that the hydrolysis of silver carp protein with Alcalase or Flavourzyme proceeded at a high rate during the initial 15 min and then slowed down thereafter (Figure 4), which indicated that maximum cleavage of peptides occurred within 15 min of hydrolysis.

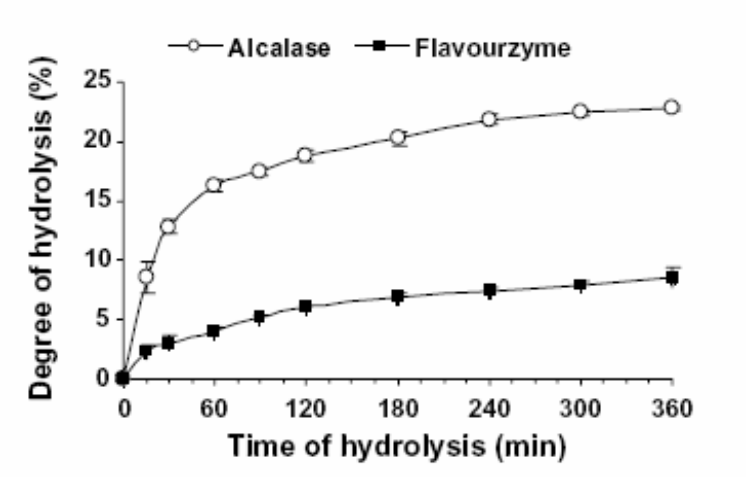

Figure 4 Effect of hydrolysis time on DH of silver carp defatted meat during hydrolysis with Alcalase and Flavourzyme at 0.5:100 (w/w) enzyme/substrate and 1:7 (w/w) substrate/water. The reaction was run at pH 8.0, 60ºC for Alcalase and pH 7.0, 50ºC for Flavourzyme, respectively.

Source : Dong et al. (2008)

4.4 Gelatin hydrolysates

Generally, gelatin is produced from by-products such as bone, skin, fin and scale. Gelatin can be converted to value-added products by enzymatic hydrolysis, which is widely applied to improve and upgrade the functional, nutritional properties of proteins and nutraceutical or bioactive peptides (Fahmi et al., 2004; Je et al., 2007). Gelatin peptides contain mainly hydrophobic amino acids and the abundance of these amino acids is associated with higher affinity to oil and better emulsifying ability (Mendis et al., 2005). The different gelatin peptide had different amino acid composition and sequences, depending on gelatin sources and enzymes used. Kim et al. (2001) prepared gelatin hydrolysate from Alaska pollack skin with serial digestions in the order of Alcalase, Pronase E, and collagenase. Mendis et al. (2005) produced peptides derived from tryptic hydrolysate of jumbo squid (*Dosidicus gigas*) skin gelatin. Lin and Li (2006) produced gelatin hydrolysate from jumbo flying squid skin with a serial hydrolysis by Properase E and pepsin. Zhao et al. (2007) prepared gelatin hydrolysate from the sea cucumber (*Acaudina molpadioidea*) sequentially with bromelain and Alcalase. Zhu et al. (2007) produced hydrolysate from puffer fish skin (*Takifugu rubripes*) with papain. Giménez et al. (2008) prepared gelatin hydrolysates obtained from sole and squid skin gelatin by using Alcalase. Yang et al. (2008) prepared cobia skin gelatin hydrolysate by using bromelain, papain, pancreatin, and trypsin. Bioactive peptides can be released by enzymatic proteolysis of gelatin and may act as potential physiological modulators of metabolism during the intestinal digestion of the diet; this protein could be good candidate as nutraceuticals (Je et al., 2007).

5. Lipid oxidation

Lipid oxidation is the process by which molecular oxygen reacts with unsaturated lipids to form lipid peroxides. The direct reaction of lipids with oxygen is spin-forbidden because the ground state of lipids is single multiplicity whereas oxygen is of triplet multiplicity. However, the spin restriction can be overcome by initiators or initiating variables such as temperature, physiological reduction of oxygen to water, photosensitizer, radiation, singlet oxygen, oxygen-transition metal complexes, or by enzymic (lipoxygenase-like) catalysis (Gordon, 2001). Lipid oxidation is known to

proceed by a free radical chain reaction mechanism involving initiation, propagation/branching, and termination stages (Monahan, 2000).

5.1 Initiation

The autoxidation of fat is thought to be initiated with the formation of free radicals. Initiation reactions take place either by the abstraction of hydrogen radical from an allylic methylene group of an unsaturated fatty acid or by the addition of radical to a double bond. The rearrangement of the double bonds resulted in the formation of conjugated diene (-CH=CH-CH=CH-), showing a characteristic UV absorption at 232-234 nm (Nakayama et al., 1994).

The formation of lipid radical (R^{o}) is usually mediated by trace metals, irradiation, light or heat (Eq. 1). Also, lipid hydroperoxide, which exists in trace quantities prior to the oxidation, breaks down to yield radicals as shown by Eqs. (2) and (3). Lipid hydroperoxides are formed by various pathways including the reaction of singlet oxygen with unsaturated lipids or the lipoxygenase-catalyzed oxidation of polyunsaturated fatty acids (Jadhav et al., 1996).

$$\text{RH + initiator} \longrightarrow \text{R}^{o} + \text{H}^{o} \quad (1)$$

$$\text{ROOH} \longrightarrow \text{RO}^{o} + \text{HO}^{o} \quad (2)$$

$$\text{2ROOH} \longrightarrow \text{RO}^{o} + \text{ROO}^{o} + \text{H}_2\text{O} \quad (3)$$

5.2 Propagation

In propagation reaction, free radicals are converted into other radicals. Propagation of free-radical oxidation processes occurs by chain reactions that consume oxygen and yield new free-radical (peroxy radicals, ROO^{o}) or by the formation of peroxides (ROOH) as in Eqs. (4) and (5) (Jadhav et al., 1996). The product R^{o} and ROO^{o} can further propagate free-radical reactions.

$$\text{R}^{o} + {}^{3}\text{O}_2 \longrightarrow \text{ROO}^{o} \quad (4)$$

$$\text{ROO}^{o} + \text{RH} \longrightarrow \text{ROOH} + \text{R}^{o} \quad (5)$$

$$\text{ROOH} \longrightarrow \text{RO}^{o} + \text{OH}^{o} \quad (6)$$

$$\text{2ROOH} \longrightarrow \text{ROO} + \text{RO}^{o} + \text{H}_2\text{O} \quad (7)$$

Lipid peroxy radicals (ROOº) initiate a chain reaction with other molecules, resulting in the formation of lipid hydroperoxides (LOOH) and lipid free radicals. The lipid hydroperoxides formed may undergo hemolytic scission to form alkoxyl (ROº) and hydroxyl radicals (OHº), which are capable of propagating further oxidation and the chain branching (Eqs. 6 and 7) (Monahan, 2000). This reaction, when repeated many times, produces an accumulation of hydroperoxides. The propagation reaction becomes a continuous process as long as unsaturated lipid or fatty acid molecules are available (Yanishlieva-Maslarova, 2001). Lipid hydroperoxide also may be formed by the reaction of an unsaturated fatty acid such as linoleic acid with oxygen in the singlet excited state or enzymatically by the reaction of lipoxygenase (Yanishlieva-Maslarova, 2001). Lipid hydroperoxides, the primary products of autoxidation, are odorless and tasteless (Jadhav et al., 1996).

5.3 Termination

A free radical is any atom with unpaired electron in the outermost shell. Free radicals are electrically neutral, and salvation effects are generally very small. Owning to the bonding-deficiency and structural unstability, radicals therefore tend to react whenever possible to restore normal bonding. When there is a reduction in the amount of unsaturated lipids (or fatty acid) present, radicals bond to one another, forming a stable nonradical compounds (Eqs. 8, 9 and 10). Thus the termination reactions lead to interruption of the repeating sequence of propagating steps of the chain reaction (Jadhav et al., 1996).

$$R^{o} + R^{o} \longrightarrow R\text{-}R \qquad (8)$$

$$R^{o} + ROO^{o} \longrightarrow ROOR \qquad (9)$$

$$ROO^{o} + ROO^{o} \longrightarrow ROOR + O_2 \qquad (10)$$

5.4 Factors influencing lipid oxidation

Many factors have been known to be associated with lipid oxidation (Nawar, 1996).

5.4.1 Fatty acid composition

The susceptibility and rate of oxidation of fatty acids in lipids depend on the number, position, geometry of double bonds and degree of their unsaturation

(Nawar, 1996). The autoxidation of major fatty acids of meat follows the order C18:0 < C18:1 < C18:2 < C18:3 (Shahidi, 1994). The following relative rates of autoxidation were determined: oleate: linoleate: linolenate = 1:12:25 (Gunstone and Hilditch, 1945) and stearate: oleate: linoleate: linolenate = 1:11:114:179 (Stirton et al., 1945). *Cis* acids oxidize more readily than their *trans* isomers, and conjugated double bonds are more reactive than non conjugated (Nawar, 1996). Autoxidation of saturated fatty acids is extremely slow; at room temperature, they remain practically unchanged when oxidative rancidity of unsaturates becomes detectable. At high temperatures, however, saturated acids can undergo oxidation at significant rates (Pryor and Porter, 1990).

5.4.2 Pro-oxidants

Enzyme such as lipoxygenase, peroxidase and microsomal enzymes, catalyze the oxidation of linoleic, linolenic and related fatty acids (Hsieh and Kinsella, 1989; Pokorny, 1999). Transition metals, particularly those possessing two or more valency states and a suitable oxidation-reduction potential between them (e.g., cobalt, copper, iron, manganese and nickel), are effective pro-oxidants. At very low concentration, < 0.1 ppm, they can decrease the induction period and increase the rate of oxidation (Oleary et al., 1992). Transition metal ions in their lower valence state (M^{n+}) react very quickly with hydroperoxides. They act as one-electron donors to form an alkoxy radical and this can be considered as the branching of the propagation step (Eq. 11). In a slow consecutive reaction, the reduced state of the metal ion may be regenerated by hydroperoxide molecules (Eq. 12) (Gordon, 2001).

$$ROOH + M^{n+} \longrightarrow RO^{o} + OH + M^{(n+1)+} \quad (11)$$

$$ROOH + M^{(n+1)+} \longrightarrow ROO^{o} + H^{+} + M^{n+} \quad (12)$$

Metals can abstract a hydrogen atom from the fatty acids themselves according to equation (Eq. 13), but the ubiquitous presence of traces of hydrogen peroxides in oils is likely to ensure that hydroperoxide decomposition is the normal initiation reaction (Gordon, 2001).

$$RH + M^{(n+1)+} \longrightarrow R^{o} + H^{+} + M^{n+} \quad (13)$$

5.4.3 Other factors

1. Oxygen concentration: Molecular oxygen behaves as a biradical by having two unpaired electron (•O-O•) in the ground state and is said to be in a triplet state (Yanishlieva-Maslarova, 2001). At very low oxygen pressure, the rate of oxidation is approximately proportional to oxygen pressure (Nawar, 1996). If the supply of oxygen is unlimited, the rate of oxidation is independent of oxygen pressure. The availability of oxygen therefore plays a critical role in determining competitive oxidative pathways (Nawar, 1996). However, the effect of oxygen concentration on rate is also influenced by other factors, such as temperature and surface area.

2. Temperature: Rate of reaction increases with increasing temperature. Temperature also influences the relationship between rate and oxygen partial pressure (Ahn et al., 1993). However, as the temperature increases, the increase in rate with increasing oxygen concentration becomes less evident, because oxygen becomes less soluble (Nawar, 1996). Orlien et al. (2005) reported that the lipid oxidation of the encapsulated and bulk stripped rapeseed oil stored at low temperature (5°C) showed the lowest oxidation, followed by intermediate temperatures (25 and 45°C) and high temperature (60°C), respectively. Baron et al. (2007) found that lipid oxidation was observed in rainbow trout fillets stored at -20ºC, whereas the fish stored at -30 and -80ºC did not show any increase in oxidation.

3. Surface area: The rate of oxidation increases in direct proportion to the surface area of the lipid exposed to air (Gil et al., 2002). Furthermore, as surface-volume ratio is increased, a given reduction in oxygen partial pressure becomes less effective in decreasing the rate of oxidation. The rate of oxidation in oil-in-water emulsions is governed by the rate at which oxygen diffuses into the oil phase (Nawar, 1996).

4. Light and sensitizer: Excitation of lipid or excitation of oxygen may occur in the presence of light and a sensitizer (such as porphyrins, bilirubin, pheophytins, riboflavin and chlorophyll) (Gordon, 2001). Fatty acids are particularly sensitive to photolytic autooxidation since they generally exhibit great absorption of UV radiation, but absorb less light in the visible spectrum. However, exposure to visible light can bring about oxidative changes in foods as well, primarily through

photosensitized oxidation, which occurs in the presence of photosensitizers (Yanishlieva-Maslarova, 2001). Photosensitizers are substances containing conjugated double bond systems which thereby have the ability to absorb light in the visible region. The absorption of light leads to an electron being excited to a higher energy level, resulting in an unstable siglet-excited state of the photosensitizer (Ahn et al., 1993). The singlet-excited state of the photosensitizer may undergo three physical processes; internal conversion, emission of light, or intersystem crossing (Yanishlieva-Maslarova, 2001). Conversion of the excited singlet state via intersystem crossing yields the triplet-excited state that is the reactive intermediate in photosensitized oxidation (Sang and Jin, 2004). Furthermore, singlet oxygen reacts much faster than triplet oxygen with the unsaturated lipid via the 'ene' reaction producing an allylic hydroperoxide via a shift of a double bond (Korycka-Dahl and Richardson, 1977).

5. Water content: In model lipid system and various fat-containing foods, the rate of oxidation is dependent strongly on water activity. In dried foods with very low moisture contents (a_w values of less than about 0.1), oxidation proceeds very rapidly. Increase in a_w to about 0.3 retards lipid oxidation by reducing metal catalysis, quenching free radicals, promoting nonenzymatic browning, and/or impeding oxygen accessibility. At higher a_w (0.55-0.85), the rate of oxidation increases again, presumably due to increased mobilization of the catalysts (Nawar, 1996). Baker et al. (2008) reported that the highest oxidation values were observed in the peanuts (high-oleic acid) held under 0.67 water activity, followed by 0.12, 0.52, 0.44 and 0.33, respectively.

6. Irradiation: Irradiation increases the initiation rate (formation of free radicals), which is due to the high energy of the light quanta, α-, β- and X-rays. The shorter the wavelength, the higher the energy and the more detrimental the irradiation effect is. The initiation rate R_i is directly proportional to the irradiation intensity, I, and the autoxidation rate W is proportional to the square root of I, e.g. $W = I^{1/2}$ (Yanishlieva-Maslarova, 2001)

6. Antioxidants

It is well known that lipid peroxidation is a major course of quality changes which affect the flavor, texture and appearance in foods (Rajapakse et al., 2005). In addition, it has been recognized that oxidative stress plays a significant role in a number of age specific diseases (Lu et al., 2008). The etiology of a range of diseases is associated with the generation of excess reactive oxygen species (ROS). A shift in the balance between ROS generation and destruction to overproduction or decreased detoxification is associated with chronic diseases (Je et al., 2004). Therefore, there is a continuing search for better and more effective antioxidants. The term antioxidant may refer to a range of compounds with a range of action of mechanisms. An antioxidant is any substance that, when present at low concentrations compared to those of an oxidizable substrate, significantly delays or prevents oxidation of that substrate (Halliwell et al., 1986).

The use of antioxidants in food products is governed by regulatory laws of the individual country or by internal standards. Even though many natural and synthetic compounds have antioxidant properties, only a few of them have been accepted as 'generally recognized as safe (GRAS)' substances for use in food products by international bodies such as the Joint FAO/WHO Expert Committee on Food Additives (JECFA) and the European Community's Scientific Committee for Food (SCF) (Miková, 2001).

6.1 Classification of food antioxidants

6.1.1 Primary antioxidants

Primary (chain-breaking) antioxidants terminate the free-radical chain reaction by donating hydrogen or electron to free radicals and converting them to more stable products. They may also interact with the lipid radicals, forming lipid-antioxidant complexes (Decker, 1998). Many of the naturally occurring phenolic compounds like flavonoids, eugenol, vanillin and rosemary antioxidant also have chain-breaking properties (Sathivel et al., 2003). Primary antioxidants are effective at very low concentrations but at higher levels they become prooxidants (Rajalakshmi and Narasimhan, 1996). A characteristic action of antioxidants of this type is that, at least in *in vitro* reactions, they produce a lag period, the so-called induction period,

which usually is proportional in duration (or 'length') to their concentration, and which continues until about 90% of the antioxidant has been destroyed. During this lag period, lipid peroxidation proceeds at a very low rate, but at the end, the oxidation continues at a rate equal to that of the unprotected lipid, or even greater (Yanishlieva-Maslarova, 2001).

6.1.2 Secondary antioxidants

Secondary or preventive antioxidants such as thiopropionic acid and dilauryl thiodipropionate function by decomposing the lipid peroxides into stable end products (Rajalakshmi and Narasimhan, 1996). Synergistic antioxidants can be broadly classified as oxygen scavengers and chelators. However, they may act as hydrogen donors to the phenoxy radical, thereby regenerating the primary antioxidant (Decker, 1998). Hence phenolic antioxidants can be used at lower levels if a synergist is added simultaneously to the food product. Oxygen scavengers such as ascorbic acid, ascorbyl palmitate, sulfites and erythobates react with free oxygen and remove it in a closed system (Rajalakshmi and Narasimhan, 1996). The synergistic effect of citric acid is attributed to metal chelation (Frankel et al., 1997). Other polyvalent acids such as tartaric, malic, gluconic, oxalic, succinic and dehydro glutaric acids, as well as sodium triphosphate and pyrophosphate also show the synergistic properties similar to those of citric acid (Yanishlieva-Maslarova, 2001). Moreover, ascorbic acid can act as a synergist with tocopherols by regenerating or restoring their antioxidant properties (Niki, 1987). Ascorbic acid and its derivatives also function as oxygen scavengers (Yanishlieva-Maslarova, 2001).

6.2 Mode of action of antioxidants in food

6.2.1 Radical scavenger

Antioxidants can retard or inhibit lipid oxidation by inactivating or scavenging free radicals, thus inhibiting initiation and propagation reaction. The chain-breaking antioxidant (AH) scavenge the free radicals such as peroxyl (ROO^{o}) and alkoxyl (RO^{o}), interrupting the propagation step and forming an antioxidant radical (A^{o}) of such a low reactivity that no further reaction with lipid can occur (Decker, 1998; Yanishlieva-Maslarova, 2001). Antioxidants can scavenge free radical

either as hydrogen donors or as electron donors that form charge-transfer complexes (Osawa, 1994).

Hydrogen donor

$ROO^{o} + AH \longrightarrow ROOH + A^{o}$

$RO^{o} + AH \longrightarrow ROH + A^{o}$

$R^{o} + AH \longrightarrow RH + A^{o}$

Electron donor

$ROO^{o} + A^{o} \longrightarrow (AH\text{-}ROO)^{o}$

$(AH\text{-}ROO)^{o} + ROO^{o} \longrightarrow$ Stable product

The free antioxidant radicals (A^{o}) may undergo additional reactions that remove radical from the system. Termination reactions of antioxidant with other free antioxidant radicals or lipid radicals can form nonradical species.

$ROO^{o} + A^{o} \longrightarrow ROOA$

$RO^{o} + A^{o} \longrightarrow ROA$

$A^{o} + A^{o} \longrightarrow AA$

Antioxidants may act as hydrogen donors to the phenoxyl radicals, which are stabilized by resonance delocalization of the unpaired electron in the aromatic ring and act further stabilized by bulky group at the ortho position as shown in Figure 5 (Shahidi and Wanasundara, 1992). Phenolic compounds such as tocopherols, flavonoids and phenolic acids, work as antioxidants by donating the hydrogen of the hydroxyl group to the lipid peroxyl radical. The radical formed from these is stabilized through delocalization of the solitary electron over the aromatic ring structure (Yanishlieva-Maslarova, 2001).

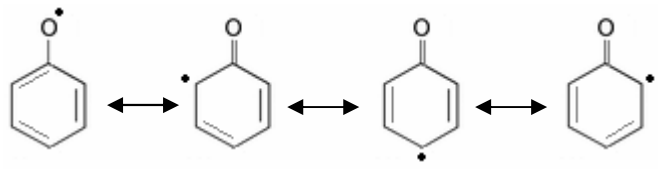

Figure 5 Delocalization of the unpaired electron in the aromatic ring of phenoxy radicals.

Source: Shahidi and Wanasundara (1992)

6.2.2 Peroxide decomposer

Some phenols, amine, thioethers, methionine, dithiopropionic acid and thiopropionic acid function by decomposing the lipid peroxide into stable end products such as alcohol, ketone and aldehyde (Namiki, 1990). Selenium and its compounds can also destroy peroxides by changing them into inactive products. They are powerful inhibitors significantly decreasing the concentration of lipid peroxides and increasing the oxidation lag time (Vinson et al., 1998).

6.2.3 Singlet oxygen quenchers

Oxygen in the environment is normally in the triplet electronic state, $^3O_2$. However, triplet oxygen can be excited by light to singlet oxygen ($^1O_2$) in the presence of a sensitizer, such as chlorophyll. Singlet oxygen reacts much faster than triplet oxygen with the unsaturated lipid via the 'ene' reaction production and allylic hydroperoxide via a shift of a double bond (Korycka-Dahl and Richardson, 1977). The singlet oxygen formed may then react with a polyunsaturated fatty acid to form a hydroperoxide, and it occurs more than 1500 times faster than the reaction between triplet oxygen and polyunsaturated fatty acid (Gordon, 2001). A carotenoids (Car) such as β-carotene, lycopene, zeaxanthin, lutein and canthaxanthin, undergoes no ultimate chemical change (physical quenching), or another route, which involves a chemical change resulting in new products (Young and Brewer, 1978). A process involving transfer of excited energy from $^1O_2$ to the carotenoids, resulting in the formation of ground state oxygen $^3O_2$ and triplet excited carotenoids (^{3}Car*). The energy is dissipated through rotational and vibrational interactions between ^{3}Car* and the solvent to recover the ground state of the carotenoids (Stahl and Sies, 1993). One molecule of β-carotene is estimated to quench up to 1000 molecules of singlet oxygen (Foote et al., 1970).

6.2.4 Lipoxygenase inhibitors

Lipoxygenase (linoleate oxygen oxidoreductase, EC 1.13.11.12) activity requires the presence of free polyunsaturated fatty acids. Lipoxygenase molecules contain one atom of iron that catalyzes the oxygenation of the 1,4-pentadiene sequence of polyunsaturated fatty acid to produce their corresponding hydroperoxide (Salas et al., 1999; Gordon, 2001). Flavonoids, phenolic acids and gallates have been shown to inhibit the lipoxygenase (Yanishlieva-Maslarova, 2001). Theaflavin

monogallate B and theaflavin digallate appeared also to be active in inhibiting soybean lipoxygenase (Xie et al., 1993).

6.2.5 Synergists

6.2.5.1 Chelating agent

Metal chelating agents may have a dramatic effect on increasing the oxidation stability through blocking the pro-oxidant metal irons, and thus limiting the formation of chain initiators by preventing metal-assisted homolysis of hydroperoxides (Yanishlieva-Maslarova, 2001). Many metal chelating substances are present in foods, the salt of phylic acid, phospholipids, oxalates, phosphoric, tartaric and malic possess pronounced chelating activities, for example, in meat products (Ang and Hamm, 1996). Amino acids and peptides are typical metal chelating agents. Histidine-containing peptides such as carnosine and anserine are thought to have the metal-chelating ability (Egorov et al., 1992). Water-soluble metal chelators, such as ethylenediamine tetraacetic acid (EDTA) and its salt, phosphate and ascorbic acid, are effective in improving the oxidative stability of aqueous food emulsion system (Frankel, 1998).

6.2.5.2 Reducing agent or oxygen scavengers

Reducing agents or oxygen scavengers function by various mechanisms. They may act as hydrogen donors to the phenoxy radicals, thereby regenerating the primary antioxidant or react with free oxygen and remove it in a closed system (Rajalakshmi and Narasimhan, 1996). Ascorbic acid and its esterified derivatives may also function as reducing agent, readily losing H+ to become dehydroascorbic acid, which also has vitamin C activity. However, vitamin C activity is lost, when the lactone ring of dehydroascorbic acid is hydrolyzed to yield diketogluconic acid (Gordon, 2001). Ascorbic acid can act as a synergist with tocopherols by generating or restoring their antioxidants properties (Niki, 1987).

6.3 Antioxidative activity of gelatin hydrolysates

Gelatins, the heterogeneous mixture of high molecular weight water-soluble protein derived from collagen, and theirs hydrolysates in general have strong antioxidant activities (Zhu et al., 2008). Gelatin hydrolysate contains free amino acids and peptides, which have been found to exhibit antioxidant activity. Gelatin

hydrolysates have been recognized to act as natural antioxidant against free radicals or lipid oxidation in food and biological system (Je et al., 2005).

6.3.1 Radical scavenging activity and reducing power

Antioxidant peptides derived from different sources have exhibited varying potencies to scavenge free radicals. Gelatin hydrolysates have been found to possess the radical scavenging activity. Gelatin hydrolysates may contain certain peptides that are electron donors and can react with free radicals to terminate the radical chain reaction (Yang et al., 2008). Mendis et al. (2005) found that peptides derived from tryptic hydrolysate of jumbo squid (*Dosidicus gigas*) skin gelatin could scavenge highly active free radicals in oxidative systems, in the order of hydroxyl and carbon-centered radicals. Cobia (*Rachycentron canadum*) skin gelatin hydrolysate is able to quench DPPH radicals and there is a good correlation (r = 0.982) between its concentrations (2–20 mg/ml) and DPPH radical scavenging effects (8.6–85.8%) (Yang et al., 2008). Peptide from puffer fish skin gelatin exhibited higher hydroxyl radical and DPPH radical scavenging activities than Vitamin C and Vitamin E (Zhu et al., 2008).

Reducing capacity of a given compound may serve as a significant indicator of its potential antioxidant activity. An electron-donating reducing agent is able to donate an electron to a free radical. As a result, the radical is neutralized and the reduced species subsequently acquires a proton from solution (Wang et al., 2008). Giménez et al. (2008) reported that both sole and squid gelatin hydrolysates showed approximately a 2-fold higher ferric iron reducing ability than the corresponding gelatins.

6.3.2 Metal chelating

Although hydrolysates from other protein sources such as soybean, egg yolk, porcine myofibrillar and potato are known to act as an antioxidant via metal ion chelation and likely prevent the lipid oxidation via metal chelating ability (Chen et al., 1996; Lu and Baker, 1986; Saiga, 2003; Wang and Xiong, 2005), there are a few reports on gelatin hydrolysate. Mendis et al. (2005) reported the low ferrous ion chelation ability of peptides derived from jumbo squid skin gelatin hydrolysate. Giménez et al. (2008) reported that sole and squid gelatin hydrolysates showed a high chelating ability, with values above 80% at 0.2 mg/ml assay concentration. Generally,

histidine-containing peptides have been found to act as metal ion chelators (Chen et al., 1998). Therefore, the absence of metal ion chelation in squid skin gelatin hydrolysate could be due to relatively lower percentage of histidine residues in peptides (Mendis et al., 2005).

6.3.3 Synergistic effect with other antioxidants

Synergistic effects of antioxidants are attributed to the formation of complexes with prooxidant metal ions that are found in most of oxidizing lipids and regeneration of exhausted antioxidants (Mendis et al., 2005). The synergistic effects of nonpeptidic antioxidants on the antioxidative activity have been demonstrated with the hydrolysates of a vegetable protein, yeast protein, and bovine serum albumin (Bishov and Henick, 1975; Hatate et al., 1990). Kim et al. (2001) reported that gelatin hydrolysates from Alaska pollack skin exhibited synergistic effects with α-tocopherol.

6.3.4 Amino acids and peptides with antioxidative activity

The levels and composition of free amino acids and peptides in hydrolysate have been found to be associated with antioxidative activities (Thiansilakul et al., 2007). Phenolic hydroxyl groups present in aromatic amino acids contribute substantially for scavenging of radicals, mostly via electron donation (Suetsuna et al., 2000). Free radical quenching has been reported to be the main antioxidative mechanism of these peptides due to the amino acids, especially Pro, Ala, Val and Leu (Mendis et al., 2005). In addition, other amino acids such as histidine, proline, alanine and leucine have been reported to contribute for scavenging of free radicals (Kim et al., 2001). Potent scavenging activities of squid gelatin peptides on hydroxyl and carbon-centered radicals could be due to non-aromatic amino acids such as proline, alanine, valine and leucine (Mendis et al., 2005). Antioxidant activity of protein hydrolysates is highly dependent on their sequence and the amino acid composition (Chen et al., 1998). Many antioxidative peptides identified include hydrophobic amino acid residues Val or Leu at the N-terminus end and Pro, His or Tyr in the sequences (Chen et al., 1996). In case of gelatin peptides, the abundance of hydrophobic amino acids in their sequences seems to be responsible for the higher antioxidant effects in comparison with other antioxidant peptides due to an increase of their solubility in lipids (Mendis et al., 2005).

Separations of peptides from protein hydrolysates have been done for analyzing the relationship between molecular weight (MW) distribution and antioxidantive activity (Je et al., 2005). The MW of peptides which possessed the high antioxidative activity is different depending upon gelatin sources and enzyme used. Gelatin hydrolysate from puffer fish skin gelatin with MW below 1 kDa exhibited the highest antioxidant activities (Zhu et al., 2008). The peptides with molecular masses below 700 Da in gelatin hydrolysates from cobia skin exhibited strong DPPH free-radical scavenging activity and lipid peroxidation inhibition (Yang et al., 2008). Kim et al. (2001) reported that gelatin from Alaska pollock skin hydrolyzed with Alcalase and Pronase E was composed of peptides ranging from 1.5 to 4.5 kDa showing high antioxidative activity. Two different peptides contained 13 and 16 amino acid residues, respectively; and both peptides contained a Gly residue at the C-terminus and the repeating motif Gly-Pro-Hyp. Mendis et al. (2005) isolated two peptides derived from jumbo squid gelatin hydrolysate, Phe–Asp–Ser–Gly–Pro–Ala–Gly–Val–Leu (880.18 Da) and Asn–Gly–Pro–Leu–Gln–Ala–Gly–Gln–Pro–Gly–Glu–Arg (1241.59 Da). Both peptides could scavenge highly active free radicals in oxidative systems (Mendis et al., 2005).

OBJECTIVES

1. To characterize proteolytic activity of pyloric caeca extract (PCE) from bigeye snapper.
2. To study the antioxidative activity of gelatin hydrolysates from bigeye snapper skin prepared using PCE, Alcalase and Neutrase with varying DHs.
3. To study the antioxidative activity of gelatin hydrolysates from bigeye snapper skin prepared using PCE in combination with Alcalase or Neutrase.
4. To isolate and characterize the antioxidative peptides in gelatin hydrolysates.
5. To study the physical property, chemical composition and sensory property of apple juice fortified with of gelatin hydrolysate.

CHAPTER 2

MATERIALS AND METHODS

1. Materials

1.1 Sample

Bigeye snapper (*Priacanthus macracanthus*) were purchased from the dock in Songkhla, Thailand. The fish off-loaded approximately 18-24 h after capture, were placed in ice with a fish/ice ratio of 1:2 (w/w) and transported to the Department of Food Technology, Prince of Songkla University, Hat Yai within 2 h. Upon the arrival, fish were washed and eviscerated manually. Viscera were excised and only pyloric caeca was collected. Pyloric caeca was then ground into powder in liquid nitrogen using a blender according to the method of García-Carreño et al. (1993). The powder was placed in polyethylene bag and stored at -20ºC until use.

Skins of bigeye snapper were collected and residual meat was removed manually. Cleaned skin was washed with tap water, cut into small pieces (0.5 × 0.5 cm) and placed in polyethylene bags. Prepared skins were stored at -20°C until use. The samples were stored not longer than 3 months of storage.

1.2 Chemicals

Ethylenediaminetetraacetic acid (EDTA), pepstatin A, soybean trypsin inhibitor (SBTI), *trans*-epoxysuccinyl-L-leucyl-amino(4-guanidino) butane (E-64), L-tyrosine, bovine serum albumin (BSA), β-mercaptoethanol, N^{α}-Benzoyl-DL-arginine-*p*-nitroanilide (BAPNA), N^{α}-*p*-Tosyl-L-arginine methyl ester hydrochloride (TAME), 2,4,6-trinitrobenzenesulphonic acid solution (TNBS), 2,2-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt (ABTS), L-leucine and 2,2-diphenyl-1-picrylhydrazyl (DPPH) were purchased from Sigma Chemical Co. (St. Louis, MO, USA). Trichloroacetic acid (TCA), calcium chloride ($CaCl_2$), Tris (hydroxymethyl) aminomethane and Folin-Ciocalteu's phenol reagent were obtained from Merck (Darmstadt, Germany). Sodium dodecyl sulfate (SDS), Coomassie Brilliant Blue R-250, G-250 and *N,N,N′,N′*-tetramethyl ethylene diamine were procured from Bio-Rad laboratories (Hercules, CA, USA). 2,4,6-Tripyridyl-s-riazine

(TPTZ), ferric chloride hexahydrate, butylated hydroxyl toluene (BHT) and potassium persulfate were purchased from Fluka Chemical Co. (Buchs, Swizerland).

1.3 Enzymes

Alcalase 2.4 L and Neutrase were provided by Novozyme (Bagsvaerd, Germany).

2. Instruments

Instruments	Model	Company/Country
Blender	MX-T2GN	National, Taiwan
Colorimeter	ColorFles	HunterLab Reston, USA
Double-beam Spectrophotometer	UV-16001	Shimadzu, Japan
Freeze dryer	Dura-Top^{TM}µp	FTS system, USA
Homogenizer	T25 basic	IKA labortechnik, Selangor, Malaysia
Magnetic stirrer	BIG SQUID	IKA labortechnik, Stanfen, Germany
Microcentrifuge	MIKRO20	Hettich Zentrifugan, Germany
Oil bath	B-490	Buchi, Flawil, Switzerland
pH meter	CG 842	Schott, Germany
Refrigerated centrifuge	RC-5B plus	Sorvall, USA
Shaker	Unimax 1010	Heidolph, Schwabach, Germany
Sonicator	Transsonic 460/H	Elma, Singen, Germany
Viscometer	DV-II+	Brookfield, USA
Water bath	W350	Memmert, Schwabach, Germany

3. Methods

3.1 Preparation and characterization of trypsin from pyloric caeca of bigeye snapper

3.1.1 Preparation of crude enzyme extract

Crude extract was prepared according to the method of Hau and Benjakul (2006) with a slight modification. Pyloric caeca powder (10 g) was homogenized with 100 ml of extracting buffer (50 mM Tris-HCl, pH 8.0 containing 10 mM $CaCl_2$) at a speed of 11200 × *g* for 2 min using a homogenizer (IKA labortechnik, Selangor, Malaysia). The homogenate was then stirred continuously for 30 min at 4°C and centrifuged by a refrigerated centrifuge at 11200 × *g* for 30 min at 4°C. The supernatant obtained was filtered using a Whatman paper No.1 (Whatman International Ltd., Maidstone, England) to remove the skimmed fat layer. The filtrate was referred to as "crude extract".

3.1.2 Ammonium sulfate precipitation

Crude extract was subjected to ammonium sulfate precipitation with 40–60% saturation according to the method of Hau and Benjakul (2006). The pellet obtained after centrifugation at 11200 × *g* for 30 min at 4°C was dissolved in 5 ml of 50 mM Tris-HCl, pH 8.0. The solution was then dialyzed against 15 volumes of extraction buffer for 12 h at 4°C with three changes of dialysis buffer. The dialysate was kept at 4°C and defined as "Pyloric caeca extract; PCE".

3.1.3 Assay for proteolytic activity

3.1.3.1 Proteinase activity

Proteinase activity of PCE was assayed using casein as a substrate according to the method of Klomklao et al. (2004). PCE (200 µl) was added into assay mixtures containing 2 mg of casein, 200 µl of distilled water and 625 µl of reaction buffer. The mixture was incubated under conditions tested for 20 min. Enzymatic reaction was then terminated by adding 200 µl of 50% (w/v) TCA. Unhydrolyzed protein substrate was allowed to precipitate for 1 h at 4 ˚C, followed by centrifuging at 5500 × *g* for 10 min. The oligopeptide content in the supernatant was determined by the Lowry assay (Lowry et al., 1951) using tyrosine as a standard. One unit of activity was defined as that releasing 1 mmol of tyrosine per min (mmol

/Tyr/min). A blank was run in the same manner, except the enzyme was added after addition of 50% TCA (w/v).

3.1.3.2 Trypsin activity

(1) BAPNA

Trypsin activity was measured according to the method of Benjakul et al. (1999) using BAPNA as a substrate. A sample (200 µl) with an appropriate dilution was added with 200 µl of distilled water and 1000 µl of 50 mM Tris-HCl, pH 8.0 containing 10 mM $CaCl_2$. To initiate the reaction, 200 µl of BAPNA (2 mg/ml) was added and mixed thoroughly. After incubation for 10 min at 25°C, 200 µl of 30% acetic acid (v/v) was added to terminate the reaction. The absorbance of reaction mixture was read at 410 nm using UV-1601 spectrophotometer. Trypsin amidase activity was then calculated using the following formula:

$$\text{Activity (U/ml)} = \frac{(A - A_0) \times \text{Final volume of the mixture (ml)} \times 1000}{8800 \times \text{Time of the reaction (min)} \times 0.2 \text{ (ml)}}$$

where 8800 $cm^{-1}M^{-1}$ is the coefficient of *p*-nitroaniline; A and A_o are A_{410} of the sample and the blank, respectively.

(2) TAME

Trypsin activity was measured by the method of Klomklao et al. (2007) using TAME as a substrate. The enzyme solution with an appropriate dilution (20 µl) was mixed with 3.0 ml of 1 mM TAME in 10 mM Tris–HCl buffer, pH 8.0 and incubated at 30°C for 20 min. Production of *p*-tosyl-arginine was measured by monitoring the increase in absorbance at 247 nm. One unit of enzyme activity was defined as the amount of enzyme that hydrolyzed 1 mM of TAME in 1 min.

3.1.4 pH and temperature profile of PCE

Activity of PCE was determined over the pH ranges of 6–11 using the different buffers containing 10 mM $CaCl_2$. Buffers used included 50 mM sodium acetate for pH 6.0; 50 mM Tris-HCl for pHs 7–9; and 50 mM glycine-NaOH for pHs 10–11. The activity was assayed at 55ºC using different substrates as previously described. For temperature profile study, the activity was determined at various

temperatures (25, 30, 40, 50, 55, 60, 65 and 70 ˚C) at optimum pH using different substrates.

3.1.5 Effect of inhibitors on PCE

Effect of various inhibitors on proteolytic activity was determined using casein, BAPNA and TAME as substrates. PCE previously dialyzed against 15 volumes of 50 mM Tris-HCl, pH 8.0 containing no $CaCl_2$ overnight at 4°C was added with an equal volume of inhibitor solutions to obtain the final concentrations designated (0.1 mM E-64, 0.1 mM and 0.001 mM pepstatin A, 2 mM EDTA, 1 mg/ml SBTI). The residual activity was measured under optimal pH and temperature. Percent inhibition was then calculated.

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

3.1.6.1 SDS-PAGE

SDS-PAGE was carried out using the method of Laemmli (1970). PCE was mixed with sample buffer (0.125 M Tris-HCl, pH 6.8, 4% SDS, 20% glycerol and 10% β-mercaptoethanol) at a ratio of 1:1 (v/v). The mixture (10 µg of protein) was loaded onto the gel made of 4% stacking gel and 10% separating gel. The electrophoresis was run at a constant current of 15 mA per gel using a Mini-Protean II cell apparatus (Bio-Rad Laboratories Inc., Richmond, CA, USA). After electrophoresis, the gel was stained with 0.1% Coomassie Brilliant Blue R-250 in 50% methanol and 7.5% acetic acid for 3 h and destained with solution containing 50% methanol and 7.5% acetic acid for 3 h. Molecular weight of protein bands was estimated by comparing with wide-range-molecular-weight standards (Sigma Chemical Co, St. Louis, MO, USA) including aprotinin (6.5 kDa), α-lactalbumin (14.2 kDa), trypsin inhibitor (20 kDa), trypsinogen (24 kDa), carbonic anhydrase, bovine erythrocytes (29 kDa), glyceraldehyde-3-phosphate dehydrogenase (36 kDa), ovalbumin (45 kDa), glutamic dehydrogenase (55 kDa), albumin (66 kDa), phosphorylase B (97 kDa), β-galactosidase (116 kDa) and myosin (200 kDa).

3.1.6.2 Substrate gel electrophoresis

Substrate gel electrophoresis was performed as described by García-Carreño et al. (1993). After electrophoresis, gels were immersed in 100 ml of 2% (w/v) casein in 50 mM Tris-HCl buffer, pH 7, for 1 h with constant agitation at

0°C to allow the casein to penetrate into the gels. Gels were then transferred to 100 ml of 2% (w/v) casein in 50 mM Tris-HCl, pH 8.5 and incubated at 55°C for 15 min to develop activity zones. The gels were then stained and destained as described previously (3.1.6.1). Development of clear zones on the blue background indicated proteolytic activity.

3.2 Preparation of gelatin from bigeye snapper skin

3.2.1 Extraction of gelatin from bigeye snapper skin

Gelatin was extracted from bigeye snapper skin according to the method of Jongjareonrak et al. (2006) with a slight modification. Skins were soaked in 0.1 M NaOH with a skin/solution ratio of 1:10 (w/v) at room temperature (25°C) with a gentle stirring. The solution was changed every 30 min for 3 times to remove non-collagenous proteins and pigments. Alkaline-treated skins were then washed with tap water until neutral or faintly basic pHs of wash water were obtained. The skins were then soaked in 0.05 M acetic acid with a skin/solution ratio of 1:10 (w/v) for 3 h at room temperature with a gentle stirring to swell the collagenous material in fish skin matrix. Acid-treated skins were washed as previously described. The swollen fish skins were soaked in distilled water (45ºC) with a skin/water ratio of 1:10 (w/v) for 12 h with a continuous stirring to extract the gelatin from skin matter. The mixture was then filtered using two layers of cheesecloth. The resultant filtrate was freeze-dried.

3.2.2 Proximate and electrophoretic analysis of gelatin from bigeye snapper skin

3.2.2.1 Proximate analysis

Moisture, protein, ash and fat of gelatin were determinated according to the method of AOAC (2000) with the analytical No. of 950.46, 992.15, 942.05 and 991.36, respectively (Appendix) and expressed on dry weight basic. Hydroxyproline content was determined according to the method of Bergman and Loxley (1963) (Appendix).

3.2.2.2 SDS–polyacrylamide gel electrophoresis (SDS-PAGE)

SDS-PAGE of gelatin was carried out using the method of Laemmli (1970) (Appendix) with 4% stacking gel and 7.5% separating gel. Calf skin

acid-soluble type I collagen (Sigma Chemical Co., St. Louis, Mo., USA) was used as a standard collagen.

3.3 Production of gelatin hydrolysate from bigeye snapper skin using different enzymes

3.3.1 Effect of hydrolysis time

Gelatin was dissolved in McIlvaine buffer (0.2 M sodium phosphate and 0.1 M sodium citrate) to obtain the protein concentration of 2%. The pH of mixture was adjusted to 8.0 for Alcalase and PCE and 7.0 for Neutrase reaction (Ren et al, 2008). The mixtures were incubated at 55°C for 10 min. The enzymatic hydrolysis was started by adding enzymes (Alcalase, PCE and Neutrase) at levels of 1 or 5% (w/w). After hydrolysis time designed (0, 5, 10, 15, 20, 25, 30, 35, 40, 60, 80, 100 and 120 min), 1 ml of sample was taken, mixed with 1 ml of 1% SDS solution (85°C) and placed in a water bath at 85°C for 15 min. The hydrolysate obtained was subjected to the determination of degree of hydrolysis (DH). Hydrolysis time providing the initial velocity was selected for further study.

3.3.2 Effect of enzyme concentration

Different amounts of various enzymes (0.1, 0.5, 1, 2 and 5% (w/w) for Alcalase and 1, 2, 5, 7.5 and 10% (w/w) for PCE and Neutrase) were added into gelatin (2%) preincubated at 55ºC for 10 min and the mixture were mixed thoroughly. After hydrolysis for the selected time (section 3.3.1), 1 ml of sample was mixed with 1 ml of 1% SDS solution (85°C) and placed in a water bath at 85°C for 15 min. DH of gelatin hydrolysate was determined. Log_{10} (enzyme concentration) vs. DH was plotted. From the regression equation, the enzyme concentrations required to hydrolyze gelatin to obtain the desired DHs (5, 10, 15, 20 and 25% for Alcalase and 5, 10 and 15% for PCE and Neutrase) were calculated.

3.3.3 Determination of degree of hydrolysis (DH)

The DH of gelatin hydrolysate was determined at different hydrolysis times according to the method of Benjakul and Morrissey (1997). Diluted hydrolysate samples (125 µl) were added with 2.0 ml of 0.2125 M phosphate buffer, pH 8.2 and 1.0 ml of 0.01% TNBS solution. The solution was mixed thoroughly and placed in a temperature controlled water bath at 50°C for 30 min in dark. The reaction was

terminated by adding 2.0 ml of 1.0 M sodium sulfite. The mixture was cooled at room temperature for 15 min. The absorbance was measured at 420 nm and α-amino acid was expressed in terms of L-leucine. The DH was calculated as followed:

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

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

3.4 Production of gelatin hydrolysate from bigeye snapper skin with antioxidative activity

3.4.1 One-step hydrolysis using various single proteinases

Hydrolysate from bigeye snapper skin gelatin with different DHs (5, 10, 15, 20 and 25% for Alcalase and 5, 10 and 15% for PCE and Neutrase) were prepared as described in section 3.3.2. After 1 h of hydrolysis, the enzymes were inactivated by placing the reaction mixture at 90°C for 15 min in a water bath. The mixture was then centrifuged at 2000 × *g* at 4°C for 10 min. Supernatant referred to as "gelatin hydrolysate" was determined for antioxidative activities.

3.4.2 Two-step hydrolysis using different proteinases

Two steps of hydrolysis using different proteinases were applied. Firstly, gelatin hydrolysate was prepared using PCE, Alcalase or Neutrase to obtain DH of 15, 25 and 15%, respectively, under optimal condition for each enzyme. Further hydrolysis was conducted using another proteinase under its optimal condition for another 1 h. Thereafter, enzymes were inactivated by heating at 90°C for 15 min in a water bath. The mixture was then centrifuged at 2000 × *g* at 4°C for 10 min and the supernatant was collected and used as gelatin hydrolysate. Hydrolysates obtained were subjected to analysis of antioxidative activity.

3.4.3 Determination of antioxidative activity

3.4.3.1 DPPH radical scavenging activity

DPPH radical scavenging activity was determined as described by Wu et al. (2003) with a slight modification. Sample (1.5 ml) was added with 1.5 ml of 0.15 mM DPPH in 50% 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 spectrophotometer. The blank was prepared in the same manner, except that distilled water was used instead of the sample. A standard curve was prepared using Trolox in the range of 10–60 µM. The activity was expressed as µmol Trolox equivalents (TE)/mg protein.

3.4.3.2 ABTS radical scavenging activity

ABTS radical scavenging activity was determined as described by Binson et al. (2008). The stock solutions included 7.4 mM ABTS solution and 2.6 mM potassium persulphate 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 of ABTS solution with 50 ml of methanol in order to obtain an absorbance of 1.1 ± 0.02 units at 734 nm using a spectrophotometer. Fresh ABTS solution was prepared daily. Sample (150 µl) was mixed with 2850 µl of ABTS solution and the mixture was left at room temperature for 2 h in dark. The absorbance was then measured at 734 nm using the spectrophotometer. A standard curve of Trolox ranging from 50 to 600 µM was prepared. The activity was expressed as µmol Trolox equivalents (TE)/mg protein.

3.4.3.3 Ferric reducing antioxidant power (FRAP)

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

3.4.3.4 Linoleic acid oxidation system

The antioxidant activity of gelatin hydrolysate in a linoleic acid model system was determined according to the method of Sakanaka et al. (2004). The hydrolysate samples were dissolved in 1.5 ml of 0.1 M phosphate buffer (pH 7.0) to

obtain concentrations of 100, 500 and 1000 ppm. Each solution (0.5 ml) was mixed with 1 ml of 50 mM linoleic acid in 99.5% ethanol, and the mixtures were stored at 40°C in dark. At regular intervals, aliquots of the mixture were taken for oxidation determination using the ferric thiocyanate method according to the method of Thiansilakul et al. (2007) (Appendix). The mixtures containing BHT at 25 or 100 ppm were used for comparative purposes. The control was prepared in the same manner except that no protein hydrolysate or antioxidants were added.

3.4.3.5 Lecithin liposome system

The antioxidative activity of gelatin hydrolysate in a lecithin liposome system was determined as described by Thiansilakul et al. (2007). Lecithin was suspended in deionized water at a concentration of 8 mg/ml by stirring with a glass rod, followed by sonicating for 30 min using a sonicator (Transsonic 460/H, Singen, Germany). Hydrolysate samples at concentrations of 100, 500 and 1000 ppm (3 ml) were added to the lecithin liposome system (15 ml). The liposome suspension was then sonicated for 2 min. To initiate the assay, 20 µl of cupric acetate (0.15 M) was added. The mixture was shaken at 120 rpm at 37°C in dark. The control and systems containing 25 or 100 ppm BHT were also prepared. At regular intervals, the oxidation of liposome was monitored by determining the thiobarbituric acid reactive substances (TBARS) (Lee and Hendricks, 1997) (Appendix) and conjugated dienes (Frankel et al., 1997) (Appendix).

3.5 Fractionation of antioxidative peptides from gelatin hydrolysate

Gelatin hydrolysate with the highest antioxidative activity was subjected to gel filtration chromatography. The hydrolysate was loaded onto a Sephadex G-25 column (2.5 × 50 cm; Bio-Rad Laboratories, Hercules, Canada). The elution was carried out with 50 mM sodium phosphate buffer (pH 7.0) at a flow rate of 1 ml/min. The 3-ml fractions were collected and their absorbance was read at 220 and 280 nm. A standard distribution was determined by chromatographing independently using the following standards; Gramicidin (1880 Da), vitamin B12 (1355 Da), flavin adenine dinucleotide (829.5 Da) and Gly-Tyr (238.25 Da). Blue dextran (2000 kDa) was used to measure the void volume of the column. Log molecular weights vs. elution volume after the void volume eluted were plotted. The fractions were subjected to analysis for their

antioxidative activities and the measurement of A_{220} and A_{280}. The fractions exhibiting the high antioxidative activity were pooled and used for further study.

3.6 Characterization of antioxidative peptides from gelatin hydrolysate

Pooled Sephadex G-25 fraction with antioxidative activity was characterized for pH and thermal stability.

3.6.1 pH stability

The fraction with highest antioxidative activity was adjusted to different pHs by mixing the fractions with the different buffers at the ratio of 1:2 (v/v). Buffers used included McIlvaine buffer (0.2 M sodium phosphate and 0.1 M sodium citrate) for pH 2-6; 50 mM Tris-HCl for pHs 7–9; and 50 mM glycine-NaOH for pHs 10–12. The mixture was allowed to stand at room temperature for 30 min. Thereafter, the pHs of the sample were adjusted to pH 7.0 using double strength Tris-HCl (100 mM). The residual antioxidative activities were determined by ABTS assay.

3.6.2 Thermal stability

The fraction with highest antioxidative activity was incubated at 80 and 100°C in the temperature controlled-water bath for 30, 60 and 90 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.

3.7 Fortification of gelatin hydrolysates in apple juice

3.7.1 Preparation of apple juice fortified with gelatin hydrolysate

Apple juice (Tipco F&B Co., Ltd., Thailand) was purchased from a local supermarket. Gelatin hydrolysates with the highest antioxidantive activity (Alcalase+PCE and Alcalase+Neutrase) were then added to the apple juice at different levels (0.1 and 0.3%, w/v). Prepared juices were subjected to analyses.

3.7.2 Study on physical properties of apple juice fortified with gelatin hydrolysate

3.7.2.1 Color measurement

Color of samples was measured by Hunter lab and reported in CIE system. L*, a* and b* parameters indicating lightness, redness/greenness and

yellowness/blueness, respectively. Color measurement was carried out in ten replicates for each treatment.

3.7.2.2 Measurement of browning intensity

Browning intensity of samples was measured according to the method of Ajandouz et al. (2001). The absorbance of samples was measured at 420 nm using a spectrophotometer (UV-16001, Shimadzu, Tokyo, Japan).

3.7.2.3 Measurement of viscosity

Viscosity of samples was determined using a viscometer (DV-II+, Brookfield, MA, USA) at a constant temperature of 25ºC. Spindle No.4 and a speed of 100 rpm were used.

3.7.3 Study on chemical composition of apple juice fortified with gelatin hydrolysate

3.7.3.1 Antioxidative activity

The samples were determined for antioxidative activities using DPPH, ABTS radical scavenging activities and FRAP assays as previously described.

3.7.3.2 pH, titratable acidity and hydroxyproline content

pH of sample was measured using a pH meter (CG 842, Scott, Mainz, Germany). Titratable acidity was measured according to the method of AOAC (2000) (Appendix). Hydroxyproline content was determined following the method of Bergman and Loxley (1963) (Appendix).

3.7.4 Sensory analysis

Hedonic 9-point scale was used (9 = like extremely, 5 = neutral, 1 = dislike extremely) to evaluate the likeness of apple juice without and with the fortification of gelatin hydrolysate. Thirty panelists with the ages of 20-25 were asked to evaluate the samples on the likeness for color, turbidity, odor, flavor, body, and the overall likeness.

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 (Steel and Torrie, 1980). SPSS statistic program (Version 10.0) (SPSS, 1.2, 1998) was used for data analysis.

CHAPTER 3

RESULTS AND DISCUSSION

1. Fractionation and characterization of proteases from bigeye snapper pyloric caeca

1.1 Fractionation of proteases

Fractionation of proteases from bigeye snapper pyloric caeca was carried out using ammonium sulfate precipitation as summarized in Table 5. Fractionation using ammonium sulfate with 40-60% saturation resulted in the highest protease recovery with the yield of 43.8%, suggesting the removal of some contaminating proteins from the crude extract. This led to an increase in purity by 3.9 folds. Some protease activities were found in the fraction obtained from ammonium sulfate precipitation at 0-40% saturation and residual fraction. Ammonium sulfate precipitation of trypsin from the pyloric caeca of bluefish (*Pomatomus saltatrix*) (Klomklao et al., 2007), bigeye snapper (*Priacanthus macracanthus*) (Hau and Benjakul, 2006), brownstripe red snapper (*Lutjanus vitta*), bigeye snapper (*Priacanthus tayenus*) and threadfin bream (*Nemipterus marginatus*) (Khantaphant and Benjakul, 2008) at 40-60% saturation resulted in an increase in specific activity by 8.3, 4.53, 5.2, 7.8 and 3.6 folds, respectively. Additionally, Kristjansson (1991), Klomklao et al. (2006a) and Lu et al. (2008) found that ammonium sulfate precipitation at 30-70% saturation of trypsin from rainbow trout (*Oncorhynchus mykiss*.), pyloric ceca, spleen of tongol tuna (*Thunnus tonggol*) and mandarin fish (*Siniperca chuatsi*) pyloric caeca resulted in 4.9, 28.1 and 3.5 folds increases in specific activity, respectively. Balti et al. (2009) reported that trypsin precipitation from the hepatopancreas of cuttlefish (*Sepia officinalis*) using ammonium sulfate at 20-60% saturation resulted in an increase in specific activity by 3.2 folds. Therefore, PCE rich in proteolytic activity could be obtained by precipitation of crude extract with 40–60% saturated ammonium sulfate. Additionally, PCE had the increased specific activity and purity.

Table 5 Fractionation of proteases in crude extract from bigeye snapper pyloric caeca

Ammonium sulfate Fractionation	Total activity* (units)**	Total protein (mg)	Specific activity (unit/mg)	Purity (fold)	Yield (%)
Crude extract	146,595	653.4	224.4	1	100
0-40% Saturation	7,273	19.3	377.2	1.7	5.0
40-60% Saturation	64,236	73.9	869.0	3.9	43.8
Residue	5,983	402.6	14.9	0.1	4.1

* Protease activity was measured using casein as a substrate. The assay was carried out at pH 8.0 and 55ºC.

** One unit of enzyme activity was defined as that releasing 1 mmol of tyrosine per min.

1.2 pH and temperature profile of PCE

Effects of pH on the proteolytic activities of PCE using different substrates are shown in Figure 6(a). The optimal pH of PCE for casein, BAPNA and TAME hydrolysis was 8.0. Loss of activity of PCE was found at very acidic and high alkaline pHs, most likely attributed to irreversible protein denaturation. Under acidic and alkaline pHs, the changes in enzyme conformation are possibly caused by charge repulsion, which is associated with a decrease in electrostatic bonds (Benjakul et al., 2003). Trypsins are generally more active at alkaline pH, with pH optima ranging from 7.5 to 10.5 and the enzyme was inactive at pH 4.0 and below (Simpson, 2000; Kurtovic et al., 2006). Pyloric caeca have been reported as a source of proteolytic enzyme, particularly trypsin or trypsin-like enzymes. Cationic trypsins from Atlantic salmon (*Salmo salar*) and chinook salmon (*Oncorhynchus tshawytscha*) trypsin which were reported to show the highest activity in the pH range of 8.5-10.5 (Outzen et al., 1996; Kurtovic et al., 2006). Trypsin obtained from Monterey sardine (*Sardinops sagax caerulea*) pyloric caeca had an optimal pH of 8.0 for BAPNA hydrolysis (Castillo-Yañez et al., 2005). Alkaline proteolytic activity from tambaqui (*Colossoma macropomum*) had the optimum pH ranging from 7.0 to 9.0 when azocasein was used as a substrate (Bezerrar et al., 2000). Trypsin from pyloric caeca of the starfish

(*Asterina pecinifera*) showed an optimal pH of 8.0 when TAME was used as a substrate (Kishimura and Hayashi, 2002). In addition, Heu et al. (1995) reported that trypsin and chymotrypsin from anchovy (*Engraulis japonica*) viscera had the optimum activity at pH 9.0 and 8.0 when casein and synthetic substrates (BAPNA and BTEE) were used as substrates, respectively. The same optimal pH was found for all substrates used. The result indicated that trypsin was the major protease in PCE. The result suggested that alkaline proteases most likely trypsin or trypsin-like enzyme were present in pyloric caeca of bigeye snapper.

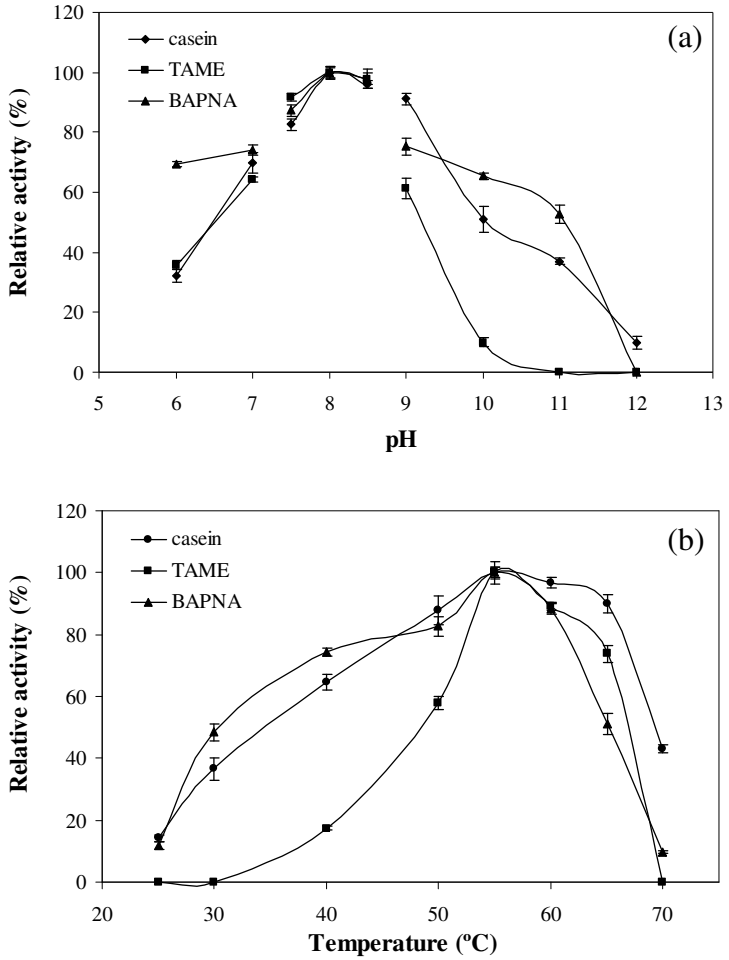

Figure 6 Effect of pH (a) and temperature (b) on the proteolytic activity of PCE from bigeye snapper using casein, N^{α}-Benzoyl-DL-arginine-*p*-nitroanilide (BAPNA) and N^{α}-*p*-Tosyl-L-arginine methyl ester hydrochloride (TAME) as substrates. Bars represent standard derivation from triplicate determinations.

Optimal temperature of proteases in PCE from bigeye snapper was 55ºC (Figure 6(b)) when casein, BAPNA and TAME were used as substrates. The activity decreased when the temperature was higher than 65ºC, probably due to the denaturation of enzymes (Hau and Benjakul, 2006). The optimal temperature of proteases in PCE was similar to those of other fish trypsins reported previously. Rate of hydrolysis of BAPNA by Atlantic cod (*Gadus morhua*) (Asgeirsson et al., 1989), bigeye snapper (*Priacanthus macracanthus*) (Hau and Benjakul, 2006) and bluefish (*Pomatomus saltatrix*) (Klomklao et al., 2007) trypsins, respectively, increased with temperature up to 55°C, despite, while rainbow trout (*Oncorhynchus mykiss.*) (Kristjansson, 1991), Japanese anchovy (*E. japonicus*) (Ahsan and Watabe, 2001) and chinook salmon (*Oncorhynchus tshawytscha*) (Kurtovic et al., 2006) trypsins had the maximum activity at 60°C. Two isozymes of trypsin purified from the viscera of Japanese anchovy (*E. japonica*) had the maximal activities at around 60ºC for hydrolysis of TAME (Kishimura et al., 2005). Alkaline serine proteinase from pyloric caeca of tambaqui (*Colossoma macropomum*) exhibited the maximal activity at 60ºC when azocasein was used as a substrate (Bezerra et al., 2001). Furthermore, Klomklao et al. (2004) reported that tuna splenic extract contained heat-activated alkaline serine proteinases as the major enzymes, which had the optimum temperature of 55ºC when casein was used as a substrate. Castillo-Yañez et al. (2005) found that the optimum temperature for Monterey sardine (*Sardinops sagax caerulea*) trypsin activity was 50ºC. However, Simpson and Haard (1984, 1985) reported temperature optima of 35 to 40ºC for Greenland cod (*Gadus ogac*) trypsin and 45°C for cunner (*Tautogolabrus adspersus*) trypsin. Anchovy trypsin exhibited the maximal activity using BAPNA as substrate at 45ºC (Heu et al., 1995). The differences in optimal temperature can be associated with the different living temperatures (Hau and Benjakul, 2006). From the result, optimal temperature was observed for all substrates used. The result indicated that PCE consisted of proteases most likely trypsin, which were active at high temperature ranges.

1.3 Effect of inhibitors on proteases in PCE

Protease inhibitors are very important tools used to classify the type of enzymes, based on active sites of enzyme tested. The effect of various inhibitors on PCE proteinase activity is shown in Table 6. When casein was used as a substrate, PCE proteinase activity was highly inhibited by 1.0 mg/ml soybean trypsin inhibitor (72.2%). Specific inhibitors of aspartic protease (pepstatin A) showed partial inhibition (12.1%). EDTA, specific for metalloprotease (Castillo-Yañez et al., 2005) had a slight inhibitory effect on proteolytic activity with approximately 6.2% inhibition. Specific inhibitor of cysteine protease (E-64) had no inhibitory effect on proteinase activity of PCE. The result indicated that major proteases in PCE were serine proteases most likely trypsin or trypsin-like enzymes. Similar results were reported in splenic extract from skipjack tuna (*Katsuwonus pelamis*), yellowfin tuna (*Thunnus albacores*), tongol tuna (*Thunnus tonggol*) (Klomklao et al., 2004) and pyloric caeca extracts of spotted mackerel (*Scomber australasicus*) (Kishimura et al., 2006b), brownstripe red snapper (*Lutjanus vitta*), bigeye snapper (*Priacanthus tayenus*) and threadfin bream (*Nemipterus marginatus*) (Khantaphant and Benjakul, 2008), in which SBTI showed the strong inhibition toward the proteolytic activity. To confirm the identity of proteases in PCE, effects of various protease inhibitors were also determined when BAPNA and TAME were used as substrates. Proteolytic activities of PCE were strongly inhibited by SBTI (98.3 and 76.2%), whereas E-64 and EDTA showed slight inhibitory activity. EDTA possibly affected the structure of the enzyme or active site conformation, leading to the reduced activity (Klomklao et al., 2007). This finding was in agreement with that of trypsin from pyloric caeca of another species of bigeye snapper (*Priacanthus macracanthus*) (Hau and Benjakul, 2006), bluefish (*Pomatomus saltatrix*) (Klomklao et al., 2007) and walleye pollock (*Theragra chalcogramma*) (Kishimura et al., 2008), in which SBTI could inhibit protease activity effectively when BAPNA and TAME were used as substrate. For trypsin from pyloric caeca of Monterey sardine (*Sardinops sagax caerulea*) and bigeye snapper (*Priacanthus macracanthus*), EDTA at the concentration of 0.25 mg/ml and 2 mM could inhibit activity by 14% and 10%, respectively (Castillo-Yañez et al., 2005; Hau and Benjakul, 2006). Novillo et al. (1999) found that activity of trypsin-like proteases from larvae of several lepidopteran species was inhibited by E-

64 when BAPNA and azoalbumin were used as substrates but were not inhibited when N^{α}-benzoyl-*L*-arginine ethyl ester (BAEE) and TAME were used as substrates. Trypsin cleaves the bond between the arginine and the *p*-nitroaniline of BAPNA or *p*-tosyl-arginine of TAME, which is easily measured using a colorimeter (Wu and Jiang, 2008). The considerable inhibition by SBTI confirmed that trypsin was the major protease in PCE. The inhibition results indicated that PCE contained serine proteases as a major proteases. Based on both synthetic substrate and specific protease inhibitor tested, it can be reconfirmed that trypsin or trypsin-like enzyme was the major protease in PCE.

Table 6 Effect of various inhibitors on the proteolytic activity* of PCE from bigeye snapper tested by different substrates

Inhibitors	Concentration	Inhibition (%)		
		Casein	BAPNA	TAME
E-64	0.1 mM	0	11.1 ± 1.6	2.4 ± 0.3
Pepstatin A	0.1 and 0.001 mM	12.1 ± 1.2	0	6.2 ± 1.2
EDTA	2 mM	6.4 ± 0.7	3.1 ± 0.5	9.1 ± 1.3
SBTI	1 mg/ml	72.2 ± 1.3	98.3 ± 1.1	76.2 ± 2.7

* All assays were carried out at pH 8.0 and 55˚C.

Mean ± SD from triplicate determinations.

1.4 SDS-PAGE and substrate gel electrophoresis

PCE contained the protein with apparent molecular weight (MW) of 24 kDa as the major band as shown in Figure 7(A). Additionally, it comprised the proteins with apparent MW of 55 and 200 kDa. When comparing protein pattern under reducing and non-reducing condition, it was noted that the band intensity of proteins with MW of 55 and 24 kDa slightly decreased. However, no marked changes were found with protein having MW of 200 kDa. The result suggested that proteins in PCE were partially stabilized by disulfide bonds. Based on substrate gel electrophoresis, two activity bands of PCE appeared as clear zones with MW of 24 and 55 kDa. Activity band with MW of 24 kDa was much larger than that of protein with MW of 55 kDa (Figure 7(B)). The result indicated that the protein band with MW of 24 kDa possessed the proteolytic activity. Generally, fish trypsin has been reported to have

the molecular weight in the range of 23–28 kDa, e.g. trypsin from capelin (28 kDa) (Hjelmeland and Raa, 1982), eel (26 kDa) (Yoshinaka et al., 1985), rainbow trout (26 kDa) (Kristjansson, 1991), carp (28 kDa) (Cao et al., 2000), Monterey sardine (25 kDa) (Castillo-Yañez et al., 2005), Japanese anchovy (24 kDa) (Kishimura et al., 2005), bigeye snapper (23.8 kDa) (Hau and Benjakul, 2006), chinook salmon (28 kDa) (Kurtovic et al., 2006), skipjack tuna (24 kDa) (Klomklao et al., 2004), walleye Pollock (24 kDa) (Kishimura et al., 2008) and cuttlefish (24 kDa) (Balti et al., 2009). The differences in MW of trypsins may be due to genetic variation among species. Furthermore, these differences might be caused by autolysis (Lu et al., 2008). The activity band, especially that with MW of 24 kDa, slightly decreased under reducing condition. The result suggested that enzyme configuration might be stabilized by disulfide bond to some extent. Serine proteases including trypsin and chymotrypsin contain three conserved disulfide bonds, which connect the loops around the substrate binding pocket (Várallyay et al., 1997).

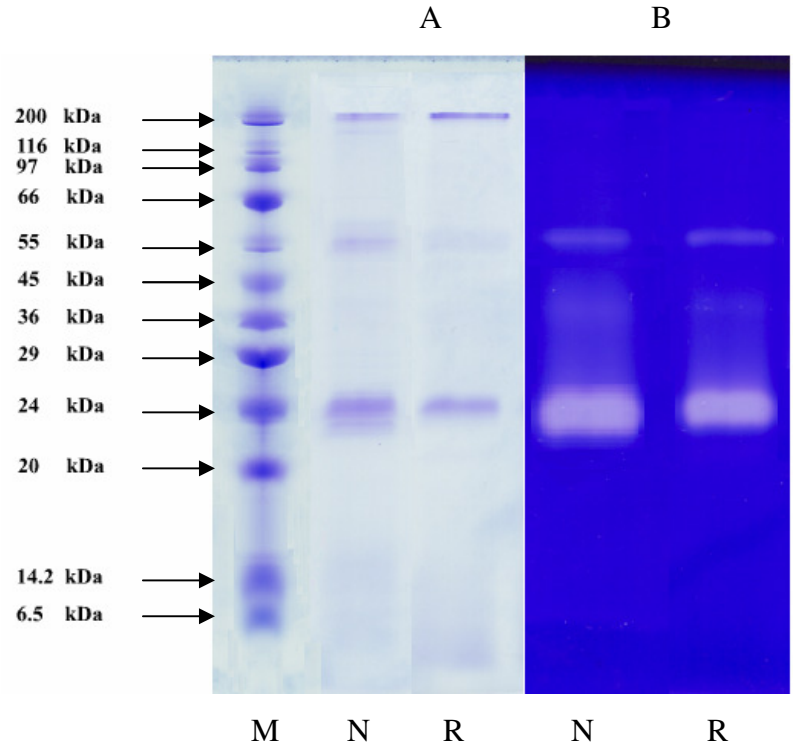

Figure 7 SDS-PAGE (A) and substrate gel electrophoresis (B) of PCE from bigeye snapper. M: marker; N: non-reducing condition; R: reducing condition.

2. Composition and protein patterns of gelatin extracted from bigeye snapper skin

2.1 Proximate composition of gelatin from bigeye snapper skin

Gelatin was extracted from bigeye snapper skin with yield of 5.8% on the basis of wet weight of fish skin. The yield of gelatin have been reported to vary among the fish species, tissue from which it is extracted and extraction process itself (Montero and Gómez-Guillén, 2000). The lower yield of gelatin extracted from bigeye snapper skin could be due to the loss of extracted collagen through leaching during the series of washing steps or due to incomplete hydrolysis of the collagen (Jamilah and Harvinder, 2002). The different yields of skin gelatin have been reported for sole (8.3%), megrim (7.4%), cod (7.2%), hake (6.5%) (Gómez-Guillén et al., 2002), red tilapia (7.81%), black tilapia (5.39%) (Jamilah and Harvinder, 2002), Baltic cod (12.3%) (Kołodziejska et al., 2004), bigeye snapper (6.5%), Dover sole (21.35%) (Giménez et al., 2005), brownstripe red snapper (9.4%) (Jongjareonrak et al., 2006), sin croaker (14.3%), shortfin scad (7.25%) (Cheow et al., 2007) and yellowfin tuna (18%) (Rahman et al., 2008). Furthermore, the high degree of cross-linking via covalent bonds caused the decrease in solubility of collagen and might lead to the lower content of extractable gelatin (Foegeding et al., 1996).

Proximate compositions of bigeye snapper skin gelatin is shown in Table 7. Extracted gelatin contained high protein content (89.77%) with low fat and ash contents, indicating on efficient removal of water and fat and minerals from the processed skin (Jongjareonrak et al., 2006). Generally, the ash content for a high quality gelatin should be lower than 0.5% (Ockerman and Hansen, 2000). Gelatin obtained consisted of 8.46% moisture content. Hydroxyproline content of bigeye snapper skin gelatin was 82.81 mg/g. Different compositions of fish skin gelatin from different sources have been reported, including brownstripe red snapper (7.6% moisture, 88.6% protein, 1.9% ash, 0.8% fat and 71.5 mg/g hydroxyproline) (Jongjareonrak et al., 2006), sin croaker (7.7% moisture, 69.2% protein, 0.1% fat and 1.5% ash), shortfin scad (11.3% moisture, 68.7% protein, 0.22% fat and 1.15% ash) (Cheow et al., 2007), Yellowfin tuna (78.1% protein and 5.6% fat) (Rahman, et al., 2008) and Nile tilapia (7.3% moisture, 89.4% protein, 0.3% lipid, and 0.4% ash)

(Songchotikunpan et al., 2008). Thus, gelatin extracted from bigeye snapper skin could be an excellent source of proteins, which could be used for hydrolysate preparation.

Table 7 Proximate composition of gelatin from bigeye snapper skin

Compositions	Contents
Protein*	89.77 ± 1.61
Moisture*	8.46 ± 0.36
Ash*	2.9 ± 0.15
Fat*	0.46 ± 0.04
Hydroxyproline**	82.81 ± 2.69

* % wet wt. ** mg/g gelatin powder.

Mean ± SD from triplicate determinations.

2.2 SDS-PAGE of gelatin from bigeye snapper skin

Protein pattern of gelatin from bigeye snapper skin are shown in Figure 8. Gelatin contained α-chains, both α_1 and α_2, as well as β-chain as the major components. Based on band intensity of α_1 and α_2–chains, gelatin obtained was derived from mother type-I collagen. Some cross-linked proteins including γ-chain were also noticeable in gelatin extracted. The presence of β-components (α-chain dimers) and higher molecular weight polymers including γ-components (α-chain trimers) was found in gelatin from sole, megrim, cod and hake (Gómez-Guillén et al., 2002). Nevertheless, no differences in protein patterns of gelatin between reducing and non-reducing conditions. The result indicated that gelatin from bigeye snapper skin contained no disulfite bond. This result was accordance with those found in skin gelatin of sole, megrim, cod, hake (Gómez-Guillén et al., 2002; Gómez-Guillén et al., 2005), bigeye snapper (Jongjareonrak et al., 2006; Nalinanon et al., 2007), pollock (Bower et al., 2006), brownstripe red snapper, threadfin bream (Khantaphant and Benjakul, 2008), channel catfish (Liu et al., 2009) and Nile tilapia (Songchotikunpan et al., 2008). From the result, the proteins with the molecule weight lower than α_2–chains was also found in gelatin. This suggested some degradation of gelatin during extraction or the residual proteins remaining after pretreatment process.

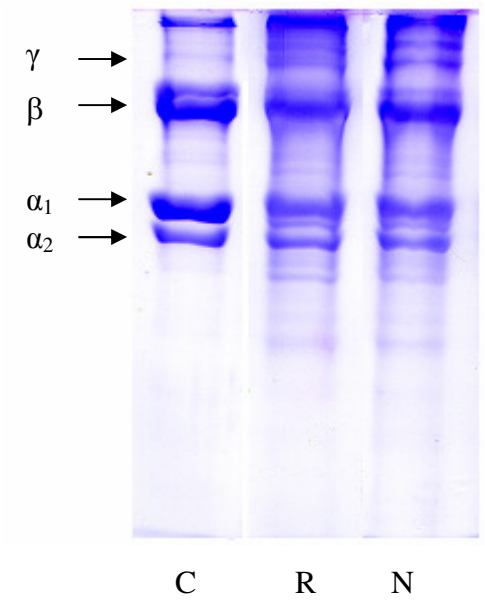

Figure 8 SDS-PAGE patterns of gelatin extracted from bigeye snapper skin. C: standard collagen type I; N: non-reducing; R: reducing condition.

3. Enzymatic hydrolysis of gelatin using different enzymes

3.1 Effect of enzyme concentrations and hydrolysis time on DH of gelatin hydrolysates

DH of gelatin hydrolysate prepared using Alcalase, Neutrase and PCE increased as the hydrolysis times increased ($p<0.05$) (Figure 9). The rapid increases in DH of gelatin hydrolysate prepared using Alcalase, Neutrase and PCE were obtained within the first 40, 10 and 40 min, respectively. Thereafter, a much lower rate of gelatin hydrolysate was noticeable for all enzymes used. The rapid hydrolysis in the initial phase indicated that a large number of peptide bonds were hydrolyzed (Shahidi et al., 1995). The subsequent decrease in hydrolysis was mainly due to a decrease in available hydrolysis sites, enzyme autodigestion and/or product inhibition (Kristinsson and Rasco, 2000). Product inhibition by compounds formed during the hydrolysis and the action of soluble peptides, which act as an effective substrate competitor for the nonhydrolyzed proteins was proposed (Rebeca, et al., 1991). Typical curve has been reported for enzymatic hydrolysis of different protein substrates such as capelin (Shahidi et al., 1995), Pacific whiting solid wastes

(Benjakul and Morrissey, 1997), salmon fillet muscle (Kristinsson and Rasco, 2000), crude rice bran protein (Adebiyi et al., 2007), yellow stripe trevally (Klompong et al., 2007), sardinelle by-products (Bougatef et al., 2008), silver carp (Dong et al., 2008) and giant sea bass (García-Gómez et al., 2009). This typical hydrolysis curve was also reported by Khantaphant and Benjakul (2008) who found the sharp increase in DH of gelatin hydrolysate prepared using pyloric caeca extracted from brownstripe red snapper, bigeye snapper and threadfin bream within the first 40, 20 and 20 min, respectively. At the same time of hydrolysis, higher DH was observed for the gelatin hydrolysate prepared using Alcalase, Neutrase and PCE with higher amounts of added enzymes. The use of 5% enzymes resulted in a greater DH of resulting hydrolysates, compared with the use of 1% enzymes. The result indicates that peptide bonds were more likely cleaved in the presence of a higher amount of enzyme (Klompong et al., 2007). However, available substrate decreases as reaction time increases (Benjakul and Morrissey, 1997).

At the same level of enzyme, Alcalase rendered the hydrolysates with higher DH than did Neutrase and PCE. DH has been used as an indicator for the cleavage of peptide bond and needed to be controlled during protein hydrolysis. The biological properties of protein hydrolysates depend on the protein substrate, the specificity of the enzyme used for the proteolysis, the conditions used during hydrolysis and the degree of hydrolysis (Bougatef et al., 2008). Degree of hydrolysis (DH), which indicates the percentage of peptide bonds cleaved (Adler-Nissen, 1979), as assessed by the TNBS assay is essential because several properties of protein hydrolysates are closely related to DH (Nielsen, 1997). From the result, the hydrolysis time of 1 h yielding the satisfactory DH was chosen for further study.

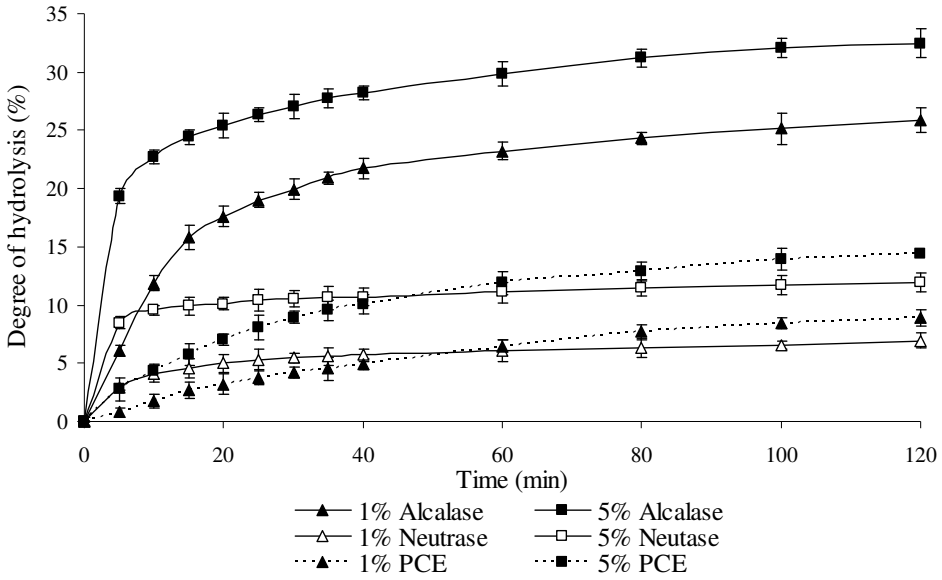

Figure 9 Change in DH of gelatin hydrolysate prepared using Alcalase, Neutrase and PCE during hydrolysis with different times. The reaction was performed at 55ºC, pH 8 for Alcalase and PCE; pH 7 for Neutrase. Bars represent the standard deviation from triplicate determinations.

3.2 Effect of enzyme concentration on DH of gelatin hydrolysates

When gelatin was hydrolyzed for 1 h with different concentrations of Alcalase, PCE and Neutrase, varying DHs were found (Figure 10). An increase in DH was generally observed with increasing enzyme concentration used (Figure 6). At the same concentration of enzyme, gelatin hydrolysate prepared using Alcalase showed appreciably higher DH than gelatin hydrolysate prepared using PCE and Neutrase respectively. The result indicated a higher hydrolytic activity of Alcalase towards gelatin from bigeye snapper, compared with that of PCE and Neutrase. Generally, Alcalase has been found to be very efficient in hydrolyzing gelatin from various species, such as Alaska pollack (Byun and Kim, 2001; Kim et al., 2001), sea cucumber (Zhao et al., 2007) and bullfog (Qian et al., 2008). Alcalase produces shorter peptides responsible for various bioactivities including antioxidant activity (Qian et al., 2008). However, Qian et al. (2008) reported that pepsin and trypsin at 1% exhibited the greater hydrolytic activities toward bullfog skin (*Rana catesbeiana Shaw*) than Alcalase, α-chymotrypsin, Neutrase and papain.

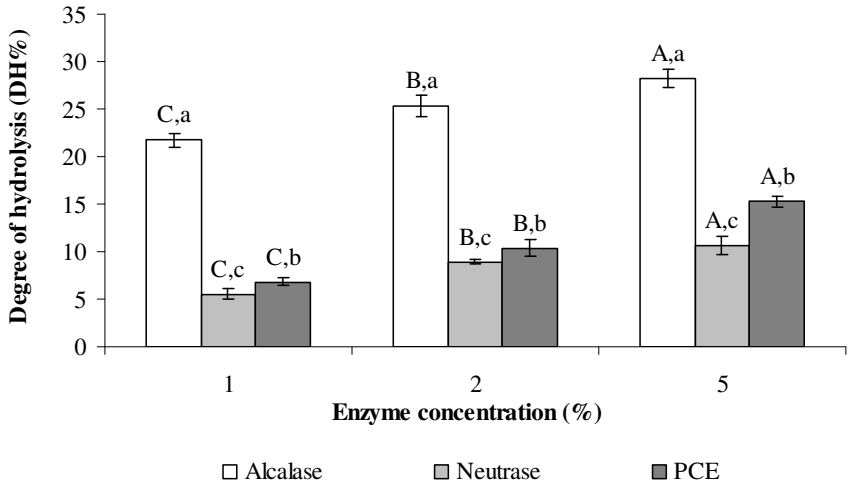

Figure 10 Degree of gelatin hydrolysate prepared using different enzymes and concentrations. Bars represent standard deviation from triplicate determinations. Different capital letters within the same enzyme indicate significant differences ($p<0.05$). Different letters within the same concentration indicate significant differences ($p<0.05$).

When $\log_{10}$ (enzyme concentration) versus DH (%) was plotted, a linear relationship was found with the correlation coefficient (R^2) of 0.9797 for Alcalase, 0.9991 for PCE and 0.927 for Neutrase (Figure 11). This result exhibited a similar linear relationship between %DH and log of enzyme concentration as reported in fish protein concentrate (Cheftel et al., 1971), Pacific whiting solid wastes (Benjakul and Morrissey, 1997), yellowfin tuna wastes (Guerard et al., 2001), yellow stripe trevally (Klompong et al., 2007) and brownstripe red snapper skin (Khantaphant and Benjakul, 2008). At the same enzyme concentration, skin gelatin treated with Alcalase showed higher DH than that treated with PCE and Neutrase, indicating a higher hydrolytic activity of Alcalase towards fish skin gelatin, compared with other proteases. From this relationship, the amount of enzyme required for gelatin hydrolysis to obtain a required DH (5, 10, 15, 20 and 25%DH for Alcalase; 5, 10 and 15%DH for PCE and Neutrase) under the optimum condition for each enzyme can be calculated. Many parameters, such as substrate, enzyme-substrate ratio, temperature and time involved in enzymatic hydrolysis generally determined the DH of hydrolysate obtained (Kristinsson and Rasco, 2000).

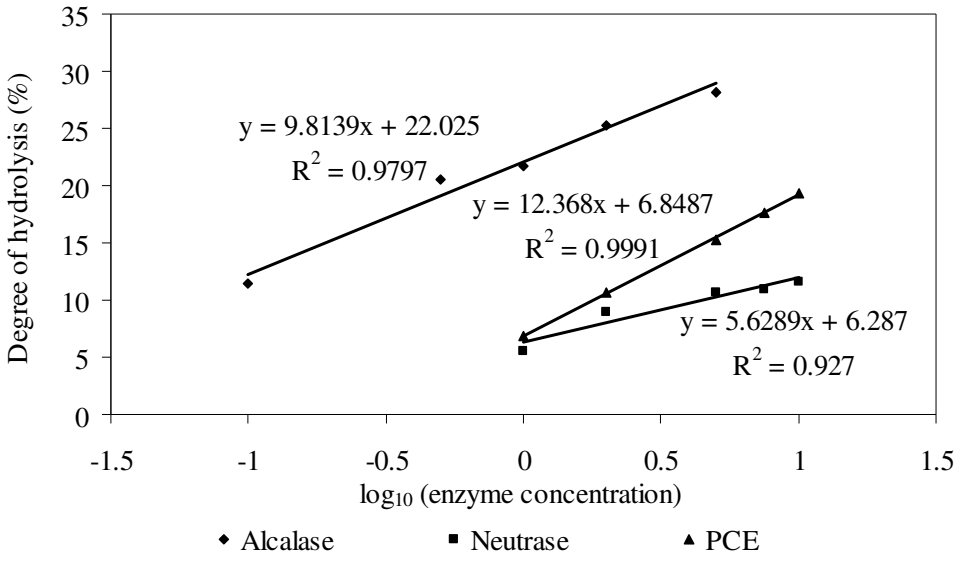

Figure 11 The relationship between $\log_{10}$ (enzyme concentration) and DH (%) in enzymatic hydrolysis of gelatin from bigeye snapper skin. The reaction was performed for 1 h at 55ºC, pH 8 for Alcalase and PCE; pH 7 for Neutrase.

3.3 Antioxidative activities of gelatin hydrolysates produced by different enzymes with various DHs

3.3.1 DPPH radical scavenging activity

DPPH radical scavenging activities of gelatin hydrolysates prepared using various enzymes with different DHs are depicted in Figure 12(a). All gelatin hydrolysates had the increases in DPPH radical scavenging activity as DHs increased ($p<0.05$). With more pronounced hydrolysis, the peptides with the higher antioxidative activity were produced. At 15% DH, hydrolysate prepared using Neutrase showed the highest activity ($p<0.05$), followed by those prepared using Alcalase and PCE, respectively. At DH of 5 and 10%, hydrolysate prepared using Alcalase had the higher activity than others. Thiansilakul et al. (2007) reported that DPPH radical scavenging activity of round scad muscle protein hydrolysate prepared using Flavourzyme and Alcalase increased with increasing DH up to 60 and 40%, respectively. Radical quenching is a primary mechanism of antioxidants to inhibit oxidative processes. DPPH is a relatively stable organic radical, thus widely used as a substrate to evaluate the efficacy of antioxidants (Sanchez-Moreno, 2002). When

DPPH encounters a proton-donating substance, such as an antioxidant, the radical is scavenged. The color is changed from purple to yellow and the absorbance is reduced (Shimada et al., 1992). The effect of antioxidants on DPPH radical scavenging was thought to be due to their hydrogen-donating ability (Binsan et al., 2008). The result revealed that the gelatin hydrolysate from bigeye snapper skin prepared using Neutrase possibly contained substances which reduced the DPPH radical to a yellow-colored compound at a highest level. Therefore, hydrolysate obtained could donate electron or hydrogen to free radicals and become more stable diamagnetic molecule (Nanjo et al., 1996), leading to the termination the radical chain reaction.

3.3.2 ABTS radical scavenging activity

As shown in Figure 12(b), with increasing DH, all gelatin hydrolysates showed the increases in ABTS radical scavenging activity ($p<0.05$). At the same DH, hydrolysate prepared using PCE showed the highest activity ($p<0.05$), followed by those prepared using Alcalase and Neutrase, respectively. Ability of hydrolysates in scavenging ABTS radicals varied with enzymes used. Hydrolysate prepared using PCE had the highest ABTS radical scavenging activity but showed the lowest DPPH radical scavenging activity ($p<0.05$), compared to hydrolysate prepared using other enzymes. The result suggested that those hydrolysates might scavenge two radicals, ABTS and DPPH, differently. It is well known that the radical system used for antioxidant evaluation may influence the experimental results, and two or more radical systems are required to investigate the radical-scavenging capacities of a selected antioxidant (Yu et al., 2002). The pre-formed radical monocation of $ABTS^{\cdot+}$ is generated by oxidation of ABTS with potassium persulphate and is reduced in the presence of such hydrogen-donating antioxidants and of chain breaking antioxidants (Binson et al., 2008). The assay of ABTS radical scavenging activity can be applied to both lipophilic and hydrophilic compounds, and has been widely used as an antioxidant activity assay (Miliauskasa et al., 2004). The result indicated that peptides produced might be different in term of amino acid composition, sequence and chain length (Khantaphant and Benjakul, 2008). Therefore, PCE was effective to produce gelatin hydrolysates containing the peptides with the highest ABTS radical scavenging activity, thereby preventing lipid oxidation via a chain breaking reaction.

3.3.3 Ferric reducing antioxidant power (FRAP)

As shown in Figure 12(c). FRAP of gelatin hydrolysates prepared using different proteases increased as DH increased ($p<0.05$). At 15% DH, the hydrolysate prepared using Neutrase showed the highest FRAP, compared with other hydrolysates ($p<0.05$). Similar FRAP was observed between hydrolysate prepared using Neutrase with 15% DH and that using Alcalase with 25% DH. Reducing power of protein hydrolysate from mackerel by autolysis and with Protease N increased gradually with increasing hydrolysis time (Wu et al., 2003). Antioxidant potential of gelatin hydrolysates was estimated from their ability to reduce TPTZ–Fe (III) complex to TPTZ–Fe (II) complex (Binson et al., 2008). In this assay, the yellow color of the test solution changes to various shades of green and blue, depending on the reducing power of each compound (Ferreira et al., 2007). From the result, gelatin hydrolysate prepared with Neutrase had capability of donating electrons to the free radical, leading to the prevention or retardation of lipid oxidation (Binson et al., 2008). Alcalase, PCE and Neutrase most likely cleaved the peptide bonds in gelatin at different positions, leading to the different peptides with varying antioxidative activity.

Therefore, the hydrolysis most likely increased the antioxidative activity of resulting hydrolysate via the enhancement of radical scavenging activity. Moreover, reducing power was increased by hydrolysis process. Differences in DPPH, ABTS radical scavenging activity and FRAP between gelatin hydrolysates from bigeye snapper skin prepared using different enzymes possibly resulted from different existing DH. Enzyme specificity toward protein substrates was associated with different peptide chain lengths and the exposure of terminal amino groups (You et al., 2008). Those factors greatly influenced the antioxidant activities of the hydrolysates, in which a wide variety of peptides with different modes of actions in inhibition of lipid oxidation was generated during hydrolysis. Size, sequence and composition of amino acids in peptides affect the antioxidative activity of protein hydrolysate (Thiansilakul et al., 2007; Wu et al., 2003). Even the same number of peptides was cleaved; the resulting peptides might be different in term of amino acid type and sequences (Rajapakse et al., 2005). Generally, hydrolysates contained peptides or proteins which were hydrogen donors and could react with the radicals to

convert them to more stable products. Those protein hydrolysates included those derived from hoki (*Johnius belengerii*) frame protein (Kim et al., 2007), round scad (*Decapterus maruadsi*) (Thiansilakulet al., 2007), silver carp (*Hypophthalmichthys molitrix*) (Dong et al., 2008), Pacific hake (*Merluccius productus*) (Samaranayaka and Li-Chan, 2008) yellow stripe trevally (*Selaroides leptolepis*) (Klompong et al., 2008), brownstripe red snapper skin gelatin (*Lutjanus vitta*) (Khantaphant and Benjakul, 2008) and grass carp (*Ctenopharyngodon idellus*) (Ren et al., 2008). Thus, gelatin hydrolysates from bigeye snapper skin with high DPPH radical scavenging activity and FRAP were prepared by using Neutrase or Alcalase to obtain DHs of 15 and 25%, respectively. To produce gelatin hydrolysate with high ABTS radical scavenging activity, PCE or Alcalase could be used to obtain DHs of 15 and 25%, respectively. Those hydrolysates were prepared and another hydrolysis using different enzymes was further conducted to increase antioxidative activity of resulting hydrolysate.

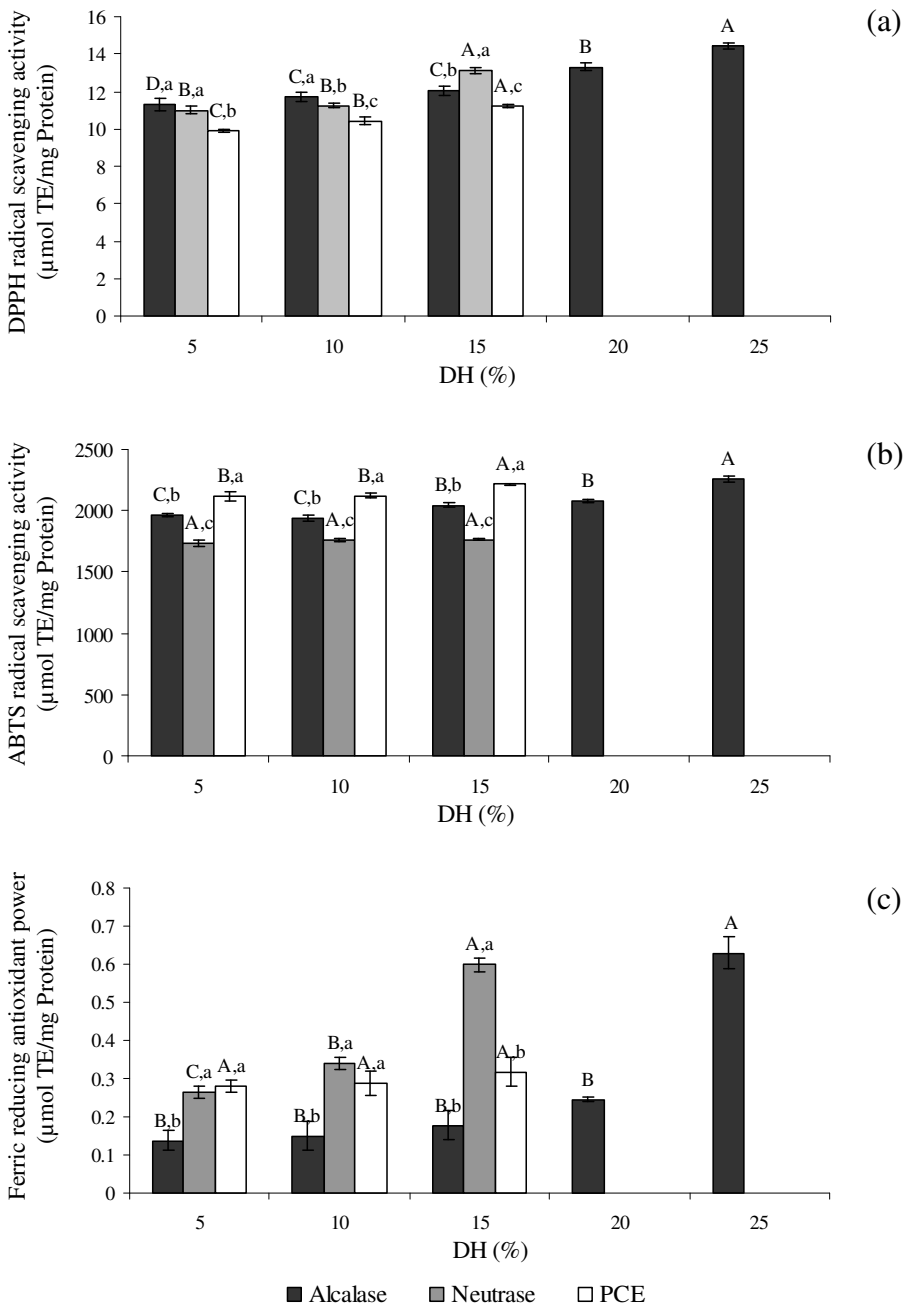

Figure 12 DPPH radical-scavenging activity (a) ABTS radical-scavenging activity (b) and ferric reducing antioxidant power (c) of skin gelatin hydrolysates prepared using Alcalase, Neutrase or PCE with different DHs. Bars represent standard deviation from triplicate determinations. Different capital letters within the same enzyme used indicate significant differences ($p<0.05$). Different letters within the same DH indicate significant differences ($p<0.05$).

3.4 Antioxidative activities on gelatin hydrolysates produced by two-step hydrolysis using different enzymes

Gelatin hydrolysates from bigeye snapper skin was hydrolyzed with two-step hydrolysis using two different enzymes including Alcalase+Neutrase, Alcalase+PCE, Neutrase+Alcalase, Neutrase+PCE, PCE+Alcalase and PCE+Neutrase. First step of hydrolysis was carried out under the optimum condition and selected DH (Alcalase = 25% DH, Neutrase = 15% DH and PCE = 15% DH). The second step was conducted with different enzymes using the optimal conditions of designated enzyme for 1 h.

Degree of hydrolysis (DH) of gelatin hydrolysates prepared using two-step hydrolysis is shown in Table 8. All gelatin hydrolysates had the increases in DH when the second step hydrolysis was conducted using another enzyme. After the second step hydrolysis, the increases in DH by 5% were obtained when compared with that found in the first step hydrolysis. The use of Alcalase for the first hydrolysis together with Neutrase or PCE for the second hydrolysis yielded the higher DH than the use of Neutrase in combination with PCE, regardless of the order of hydrolysis. From the result, gelatin hydrolysates prepared by two-step hydrolysis could increase DH and might produce more peptides with antioxidantive activity than one-step hydrolysis.

Table 8 Degree of hydrolysis of gelatin hydrolysate prepared using two-step hydrolysis

First step	Second step	%DH
Alcalase	-	$25.49 \pm 0.17^{B\dagger}$
	Neutrase	$29.96 \pm 0.15^{A,a\ddagger}$
	PCE	$30.02 \pm 0.47^{A,a}$
Neutrase	-	15.24 ± 0.23^{C}
	Alcalase	$29.46 \pm 0.55^{A,a}$
	PCE	$20.47 \pm 0.49^{B,b}$
PCE	-	15.14 ± 0.27^{C}
	Alcalase	$29.82 \pm 0.67^{A,a}$
	Neutrase	$21.32 \pm 0.21^{B,b}$

† Means ± SD from triplicate determinations.

‡ Different capital letters within the same first-step enzyme indicate significant differences ($p<0.05$). Different letters within the hydrolysates derived from two-step hydrolysis indicate significant differences ($p<0.05$).

3.4.1 DPPH radical scavenging activity

DPPH radical scavenging activity of gelatin hydrolysates from bigeye snapper skin using the combination of two enzymes is shown in Figure 13(a). Gelatin hydrolysate prepared using combination of two enzymes showed the increased DPPH radical-scavenging activities except hydrolysates prepared using Alcalase+PCE and Neutrase+PCE which showed no increase in activity ($p>0.05$), compared with hydrolysate prepared using Alcalase and Neutrase alone. Hydrolysate produced by the combination of Alcalase and Neutrase (N+A and A+N) showed the highest DPPH radical scavenging activity ($p<0.05$). The result suggested that peptides generated from the combined hydrolysis using two different enzymes acted as a good electron donor and could react with free radicals to terminate the radical chain reaction. Wu et al. (2003) reported that the DPPH radical scavenging activities of mackerel (*Scomber australasicus*) protein hydrolysates obtained by both autolysis and Protease N increased gradually with increasing hydrolysis time. Gelatin hydrolysate prepared by two-step hydrolysis had more active amino acids or peptides, which could react with free radicals to form more stable products.

3.4.2 ABTS radical scavenging activity

ABTS radical scavenging activity of gelatin hydrolysate prepared by combined enzymes is shown in Figure 13(b). Hydrolysates exhibited ABTS radical scavenging activity differently, depending on the enzymes used. For hydrolysate prepared using Alcalase for the first hydrolysis, the use of Neutrase for the second step of hydrolysis resulted in the lower activity, whereas the use of PCE in combination yielded the hydrolysate with increased activity ($p<0.05$). For hydrolysate using Neutrase for the first step of hydrolysis, the use of both Alcalase and PCE as the second step could enhance the activity of resulting hydrolysate. The use of Alcalase to further hydrolyze the hydrolysate firstly prepared using PCE resulted in the increased activity, but the use of Neutrase had no impact on ABTS radical scavenging activity of resulting hydrolysate ($p>0.05$). The result suggested that peptides produced during hydrolysis by different enzymes could be varied in term of ABTS radical scavenging activity.

3.4.3 Ferric reducing antioxidant power (FRAP)

FRAP of gelatin hydrolysates prepared using different combinations of enzymes is shown in Figure 13(c). Hydrolysate prepared by the first hydrolysis with Alcalase followed by Neutrase showed the highest FRAP ($p<0.05$) and FRAP was greater than that of hydrolysate prepared using only Alcalase ($p<0.05$). However, PCE had no effect on FRAP of hydrolysate when it was used for the second step of hydrolysis. For hydrolysate prepared using Neutrase, the uses of Alcalase or PCE for the second hydrolysis resulted in the increased FRAP, but the former yielded hydrolysate with higher FRAP than the latter ($p<0.05$). On the other hand, the use of Alcalase and Neutrase for the second hydrolysis of hydrolysate firstly prepared using PCE led to the decreased FRAP ($p<0.05$). The differences in FRAP might be governed by different peptides in hydrolysate. A number of studies had already shown that the antioxidant activity of hydrolysates was depending on their molecular weight distribution (Wu et al., 2003). Kim et al. (2001) prepared hydrolysate with antioxidative activity from Alaska pollack skin gelatin using serial digestions in the order of Alcalase, Pronase E, and collagenase. From the result, gelatin hydrolysate from bigeye snapper skin most likely contained certain peptides that were electron donors to free radicals. This mode of action contributes to the termination of the radical chain reaction.

Due to the high DPPH radical scavenging activity and FRAP of hydrolysate prepared using serial hydrolysis including Alcalase and Neutrase (A+N) and high ABTS radical scavenging activity of hydrolysate preparing using serial hydrolysis involving Alcalase and PCE (A+PCE), both A+N and A+PCE were selected for study in different lipid oxidation systems.

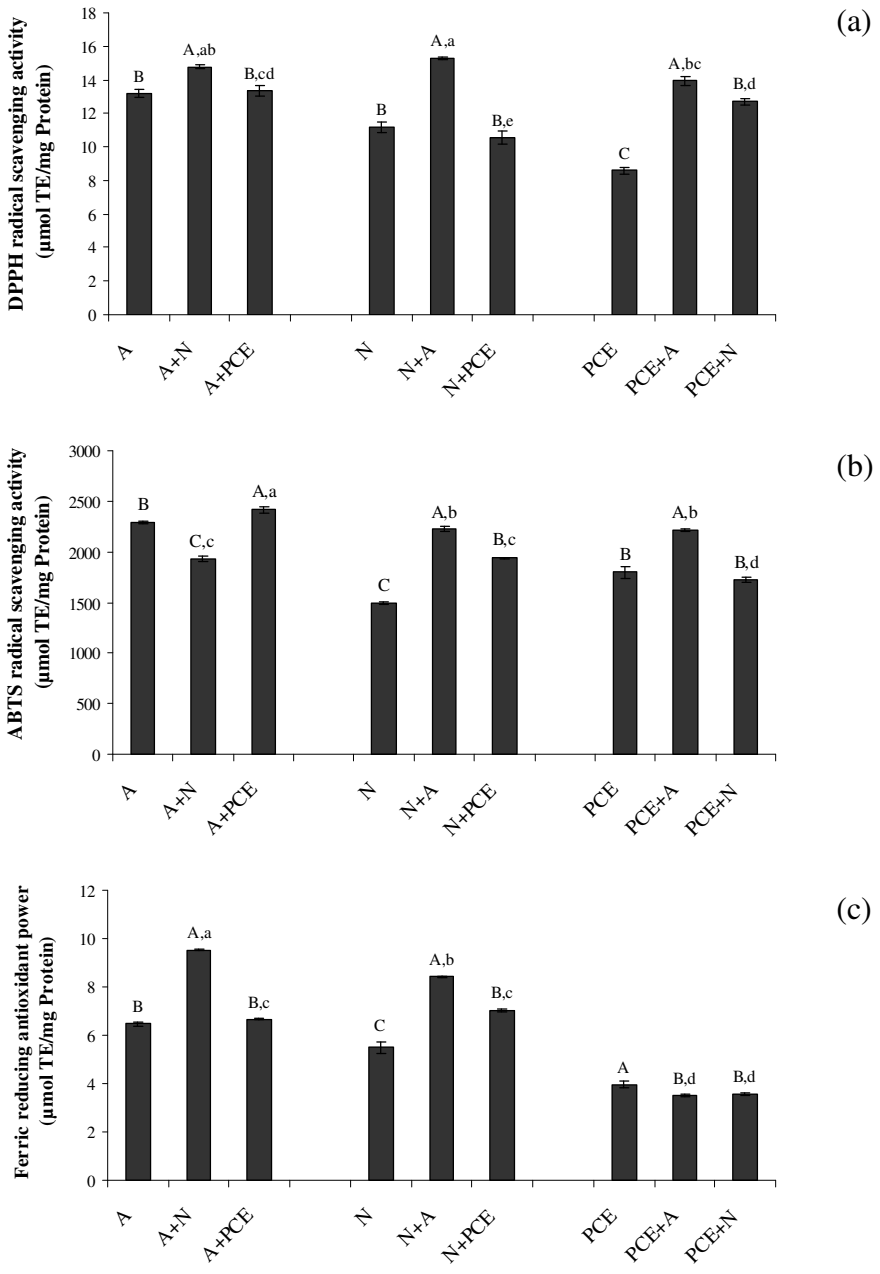

Figure 13 DPPH radical-scavenging activity (a) ABTS radical-scavenging activity (b) and ferric reducing antioxidant power (c) of skin gelatin hydrolysates prepared using two-step hydrolysis with different enzymes. A: Alcalase; N: Neutrase; PCE: pyloric caeca extract. Bars represent standard deviation from triplicate determinations. Different capital letters within the same first-step enzyme indicate significant differences ($p<0.05$). Different letters within the hydrolysates derived from two-step hydrolysis indicate significant differences ($p<0.05$).

3.4.4 Antioxidative activity in linoleic oxidation system

Both gelatin hydrolysates prepared using Alcalase+Neutrase or Alcalase+PCE showed antioxidative activity in linoleic oxidation system. The antioxidant activity of gelatin hydrolysate was determined using the thiocyanate method, in comparison with BHT. Linoleic acid, an unsaturated fatty acid, is usually used as a model compound in lipid oxidation and antioxidation-related assays of emulsion system, in which carbon-centered, peroxyl radicals and hydroperoxides are involved in the oxidation process (Burton and Ingold, 1986). Lipid peroxidation is thought to process via radical-mediated abstraction of hydrogen stoms from methylene carbons in polyunsaturated fatty acids (Rajapakse et al., 2005). Hydroperoxides are highly unstable and react readily with ferrous ions to produce ferric ions. The resulting ferric ions are detected using thiocyanate ion as the chromogen, which shows the optimal wavelength at 500 nm (Mihaljevic et al., 1996). Inhibition of oxidation of linoleic acid by gelatin hydrolysates prepared using Alcalase+Neutrase or Alcalase+PCE were monitored upon incubation at 40ºC for 10 days as shown in Figure 14. A longer induction period indicates a stronger antioxidant activity. During incubation, the system containing gelatin hydrolysate prepared using Alcalase+PCE or Alcalase+Neutrase at all concentrations lowered the oxidation, compared with that of the control ($p<0.05$). Additionally, gelatin hydrolysate exhibited antioxidant activity in dose dependent manner. However, the system containing 25 and 100 ppm BHT had the lowest oxidation, in comparison with those with gelatin hydrolysate. Gelatin hydrolysate prepared using Alcalase+Neutrase at 100 ppm showed the lower inhibitory activity, compared with 500 and 1000 ppm. At a concentration of 100 ppm, hydrolysate prepared using Alcalase+PCE showed the higher antioxidative activity than that using Alcalase+Neutrase as evidenced by the lower increase in A_{500}. From the result, the antioxidative activity of gelatin hydrolysate was possibly attributed to the ability of peptides to interfere with the propagation cycle of lipid peroxidation, thereby slowing radical mediated linoleic acid oxidation (Thiansilakul et al., 2007). Antioxidant activity of bigeye snapper skin gelatin hydrolysates might not be attributed to a single antioxidant mechanism. As observed in many studies, antioxidants are chain-breakers of free radical mediated lipid peroxidation (Mendis et al., 2005; Rajapakse et al., 2005; Rajapakse et al.,

2006). Antioxidative peptide with 5-16 amino acid residues could inhibit auto-oxidation of linoleic acid (Chen et al., 1995). Additionally, peptides from Alaska pollack skin (Kim et al., 2001), jumbo squid (*Dosidicus gigas*) skin gelatin (Mendis et al., 2005) and bullfrog skin (Qian et al., 2008) also showed inhibitory activity in the linoleic acid model system. Oil soluble radicals that generate during oxidation attack linoleic acid directly and hence hydrophobic peptides can protect linoleic acid easily by donating photons to hydrophobic peroxyl radicals (Mendis et al., 2005). Antioxidative activity of peptide or protein is dependent on molecular size and properties such as hydrophobicity and electron transferring ability of amino acid residues in the sequence (Qian et al., 2008). To exert lipid peroxidation inhibitory activity in this system, the hydrophobic property of gelatin hydrolysate may have played an important role in exerting high affinity to linoleic acid. Many antioxidative peptides included hydrophobic amino acid residue, valine, or leucine at the N-terminus or residues existing in the peptides could facilitate the interaction with fatty acids. Thus the antioxidative activity of peptides depends on the constituents amino acids (Chen et al., 1995; Kim et al., 2001). Kim et al. (2001) reported that gelatin hydrolysate from Alaska pollack skin prepared using Alcalase followed by Pronase E showed the highest antioxidative activity which exhibited about 58% inhibition of linoleic acid peroxidation. Mendis et al. (2005) found that squid gelatin hydrolysate was rich in hydrophobic amino acids (>51%) and abundance of these amino acids was expected to exert high affinity to linoleic acid. Kim et al. (2007) reported that antioxidant peptide from hoki (*Johnius belengerii*) frame protein consist 44% hydrophobic amino acids in the sequence of antioxidant activities peptide. Therefore, it was suggested that gelatin hydrolysate of bigeye snapper skin contained peptides which could interact with lipid molecules and were able to scavenge the radicals generated or to donate electron to lipid derived radicals.

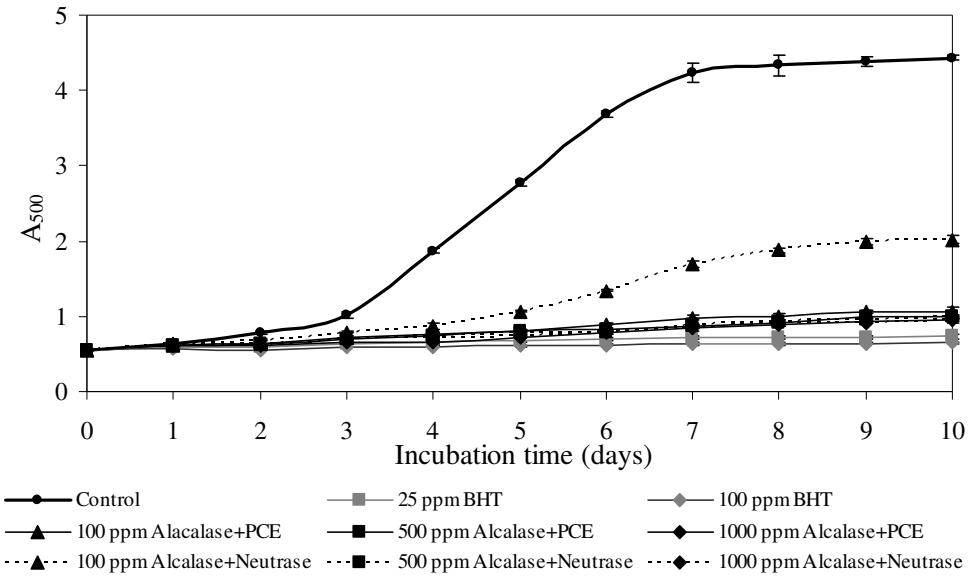

Figure 14 Effect of gelatin hydrolysates prepared using Alcalase+Neutrase or Alcalase+PCE at various amounts on A_{500} of linoleic acid system in comparison with the control, 25 and 100 ppm BHT. Bars represent standard deviation from triplicate determinations.

3.4.5 Antioxidative activity in lecithin liposome system

Gelatin hydrolysate prepared using Alcalase+Neutrase or Alcalase+PCE with different concentrations affected the oxidation of lecithin liposome system differently as indicated by the different conjugated diene and TBARS values (Figure 15). Only slight increases in conjugated diene and TBARS values of liposome system containing 100 ppm BHT were observed throughout the incubation period of 36 h. The concentration of conjugated diene in all samples significantly increased, followed by the decrease. The rate of increase varied with the hydrolysates and concentrations used. The formation of conjugated diene occurs at the early stages of lipid oxidation (Frankel et al., 1997) and hydroperoxides are expected to decompose to secondary products. The decrease or reaching a stagnant level in conjugated diene was generally accompanied by an increase in TBARS (Peña-Ramos and Xiong, 2003). Both gelatin hydrolysates could inhibit the early stage lipid oxidation (formation of conjugated diene or hydroperoxide) as well as retard propagation of the oxidation process (degradation of hydroperoxide to TBARS) (Klompong et al., 2008). Gelatin

hydrolysate prepared using Alcalase+Neutrase was generally more effective in inhibiting the lipid oxidation in lecithin liposome system than was gelatin hydrolysate prepared using Alcalase+PCE as shown by the lower conjugated diene formation throughout the incubation period of 36 h. TBARS in liposome systems without antioxidants (the control) increased markedly after incubation for 6 h at 37ºC. An increase in TBARS values of liposome system containing both hydrolysates was lower than the control and the efficiency in retardation of lipid oxidation was dependent on concentration used. For control and system added with 100 ppm hydrolysates, the decrease in TBARS was found after 24 h of incubation, while systems containing hydrolysate at 500 ppm or hydrolysate prepared from Alcalase+PCE at 1000 ppm had the decreases in TBARS after 30 h. In general, liposomes may be appropriate lipid models to evaluate antioxidants for both food and lipoprotein particles containing phospholipids (Frankel et al., 1997). These results were in agreement with Klompong et al. (2007) who reported that protein hydrolysates from yellow stripe trevally (*Selaroides leptolepis*) at 200 ppm retarded the formation of conjugated diene and TBARS in lecithin liposome system. Polar or hydrophilic domains of peptide might show affinity toward the polar surface of liposome. This led to the high effectiveness in acting as antioxidant at the interface of liposome system. From the result, gelatin hydrolysate from bigeye snapper skin functioned as antioxidants in lipid emulsion and liposome systems and could be a suitable natural antioxidant for preventing the oxidation of polyunsaturated fatty acids.

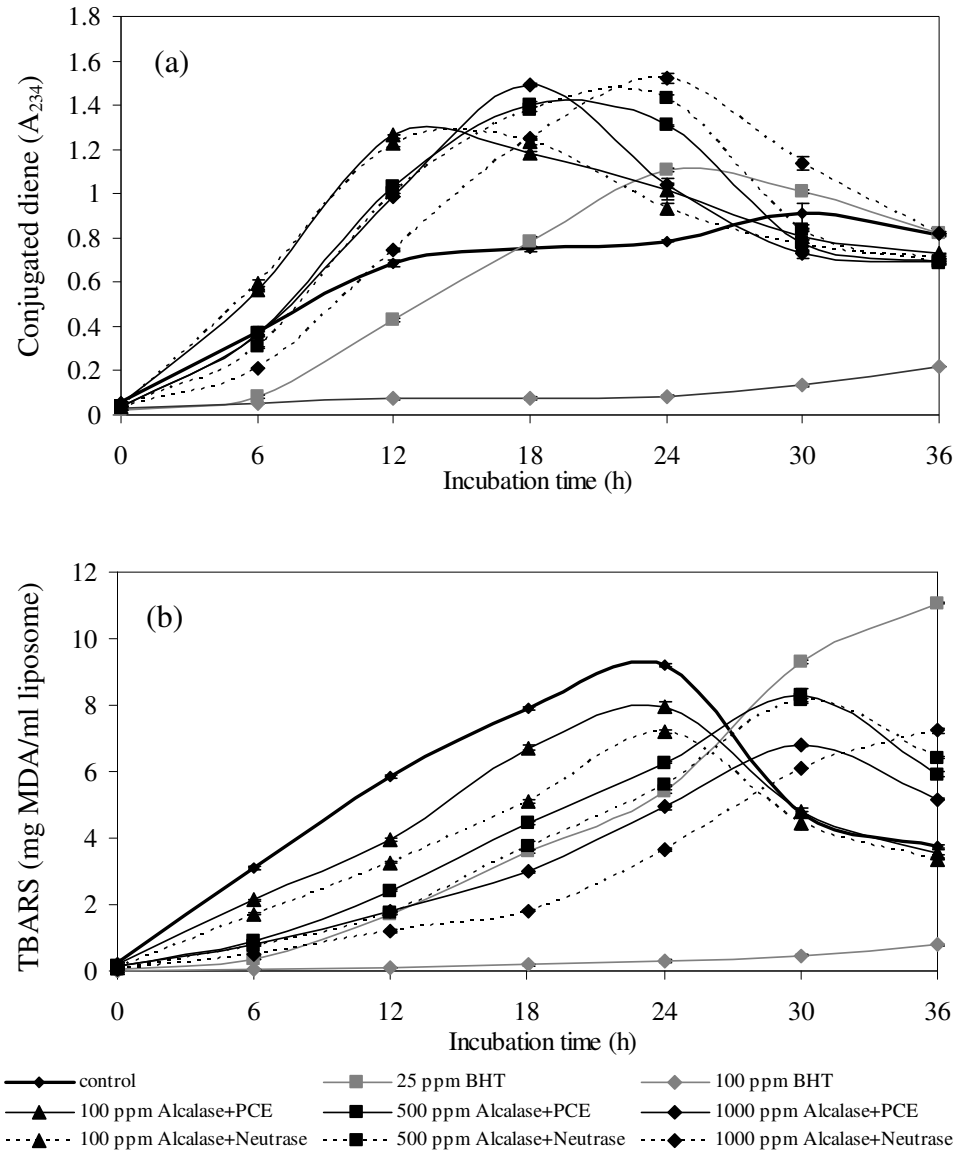

Figure 15 Effect of gelatin hydrolysates prepared using Alcalase+Neutrase or Alcalase+PCE at various amounts on the formation of conjugated diene (a) and thiobarbituric acid reactive substances (TBARS) (b) in lecithin liposome system in comparison with the control, and system containing 25 or 100 ppm BHT. Bars represent standard deviation from triplicate determinations.

4. Fractionation of antioxidative peptides from bigeye snapper skin gelatin hydrolysate

Gelatin hydrolysate prepared using Alcalase+Neutrase or Alcalase+PCE were fractionated by Sephadex-G25 gel filtration chromatography. A_{220} (Figure 16(a)) indicating peptide bond, and A_{280} (Figure 16(b)) representing peptides, proteins or amino acids with aromatic ring (Amarowicz and Shahidi, 1996) were monitored. Both hydrolysates had two peaks of A_{280}, reflecting the presence of high and low MW peptides or proteins. A peak of A_{220} was found in both hydrolysates, revealing the presence of peptide bonds in hydrolysate. However, the peak height varied with the enzyme used for hydrolysate preparation. The result suggested that there was the difference in amount and size of proteins or peptides between different hydrolysates.

ABTS radical scavenging activity of fractions from gelatin hydrolysate prepared using Alcalase+Neutrase and Alcalase+PCE were investigated. As shown in Figure 16(c), fraction containing peptides with aromatic ring (MW of 1.7 kDa) of both hydrolysates showed the highest antioxidative activity. Several reports suggested that phenolic hydroxyl groups present in aromatic amino acids contribute substantially for scavenging of radicals via acting as potent electron donors (Suetsuna et al., 2000). In general, gelatin peptides rich in glycine and proline are considered as effective radical scavengers, because they can donate protons easily to electron deficient radicals (Kohen et al., 1988). It has been known that many antioxidative peptides included hydrophobic amino acid residue, valine, phenylalanine or leucine at the N- and C-terminul of the peptides (Chen et al., 1995). Moreover, hydrophilic-hydrophobic partitioning in the sequence would play a role for the higher antioxidant properties (Mendis et al., 2005). In addition, the amino acid sequence of peptide might be responsible for its antioxidant activities, which may exceed the importance of having special amino acid such as histidine, metionine, proline, alanine, leucine or cysteine in their sequences of the peptide (Kim et al., 2001; Ren et al., 2008). Not only the presences of some favorable amino acids, but also their correct positioning in the peptide sequence are trivial to the antioxidative activity (Chen et al., 1996). Suetsuna et al. (2000) reported that leucine could enhance antioxidant activity once it presents in the C-terminus. Kim et al. (2001) isolated peptides from gelatin hydrolysate of

Alaska pollack skin ranging in size from 1.5 to 4.5 kDa. Peptides contained of 13 and 16 amino acid residues where a Gly residue is located at the C-terminus of repeating motif Gly-Pro-Hyp exhibited antioxidative activity. Wu et al. (2003) reported that the peptides from mackerel (*Scomber austriasicus*) with a molecular weight of approximately 1400 Da showed a stronger *in vitro* antioxidant activity than those of the 900 and 200 Da peptides. Mendis et al. (2005) reported that peptides derived from tryptic hydrolysate of jumbo squid (*Dosidicus gigas*) skin gelatin with MW of 880.18 Da and 1241.59 Da could scavenge highly active free radicals in oxidative systems, in the order of hydroxyl and carbon-centered radicals. Je et al. (2005) found that purified peptides with a molecular weight of less than 1000 Da from Alaska pollack (*Theragra chalcogramma*) frames proteins showed the strongest antioxidant activity among all the hydrolysate fractions. Hydrolysate from hoki (*Johnius belengerii*) frame (Kim et al., 2007), protein bullfrog (*Rana catesbeiana Shaw*) (Qian et al., 2008), cobia (*Rachycentron canadum*) (Yang et al., 2008) and puffer fish (Zhu et al., 2008) skin contained peptides having molecular weight of 1801 Da, 1487 Da, below 700 Da and below 1000 Da, respectively, showed the antioxidant activities via both lipid peroxidation inhibition ability or free radical scavenging capacity. Additonally, the hydrophobic fractions with MW of 966.3 Da (Pro-Ser-Lys-Tyr-Glu-Pro-Phe-Val) obtained from grass carp muscle hydrolysates had more contribution to the observed antioxidant activities of the hydrolysates (Ren et al., 2008). From the result, gelatin hydrolysate from bigeye snapper skin most likely contained certain peptides that were electron donors or could scavenge free radicals to terminate the radical chain reaction.

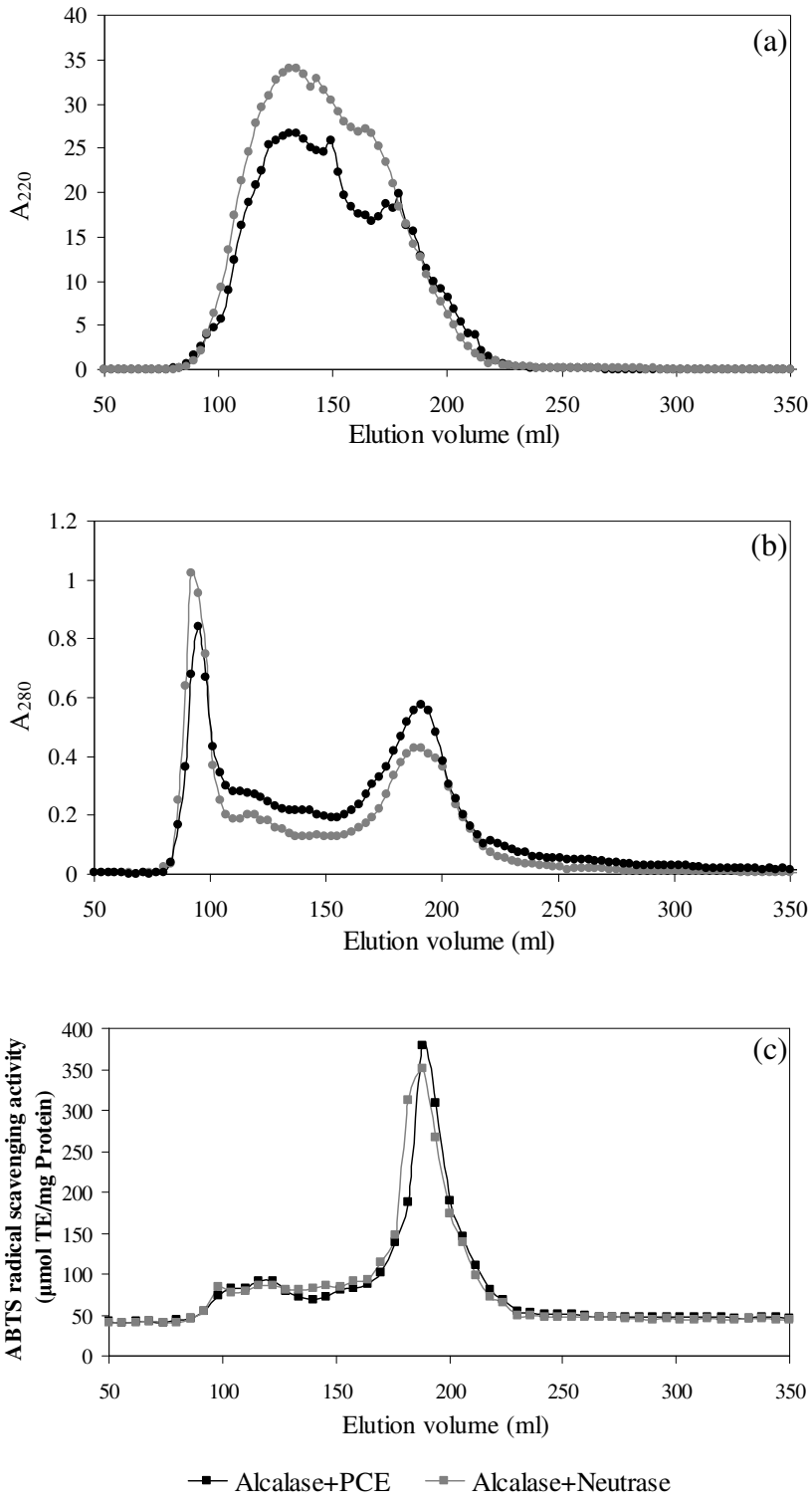

Figure 16 Separation of antioxidative peptides fractions from gelatin hydrolysates prepared using Alcalase+Neutrase or Alcalase+PCE by Sephadex G-25. (a): A_{220}; (b): A_{280}; (c): ABTS radical scavenging activity.

5. pH and thermal stability of antioxidative fractions from gelatin hydrolysates

The influences of pH on the stability of antioxidative fractions from gelatin hydrolysate prepared using Alcalase+PCE and Alcalase+Neutrase are shown in Figure 17(a). ABTS radical scavenging activities of antioxidative fractions of gelatin hydrolysate remained constant when subjected to the pH in the range of 2-12. At very acidic and alkaline pH, both fractions (Alcalase+PCE and Alcalase+Neutrase) had the slight decrease in ABTS radical scavenging activity. At pH 7.0, both fractions exhibited the highest activity ($p<0.05$). Water soluble fraction from Mungoong showed high stability over the wide pH ranges (2–11) (Binson et al., 2008). This result revealed that peptides from gelatin hydrolysate prepared using Alcalase in combination with PCE or Neutrase were stable in both acidic and alkaline pHs. This was probably associated with the small size of peptide. As a consequence, fractions could be applied as a source of antioxidative peptides in any food or drink possessing the acidic or alkaline pHs without lowering the antioxidative activity.

Thermal stability of antioxidative fractions of gelatin hydrolysates prepared using Alcalase+PCE and Alcalase+Neutrase as monitored by ABTS radical scavenging activities are shown in Figure 17(b). From the results, the antioxidative activities of both fractions were stable when heated up to 80 and 100ºC. However, the activity slightly decreased when heated up to 100ºC for 90 min where activities of more than 95% remained. This result was in agreement with Binson et al. (2008) who reported that antioxidants in the water extract from Mungoong showed high stability when temperature increased up to 100ºC, in which the activity of more than 80% remained. High thermal stability of peptide in gelatin hydrolysate could be of importance for application in thermally processed foods.

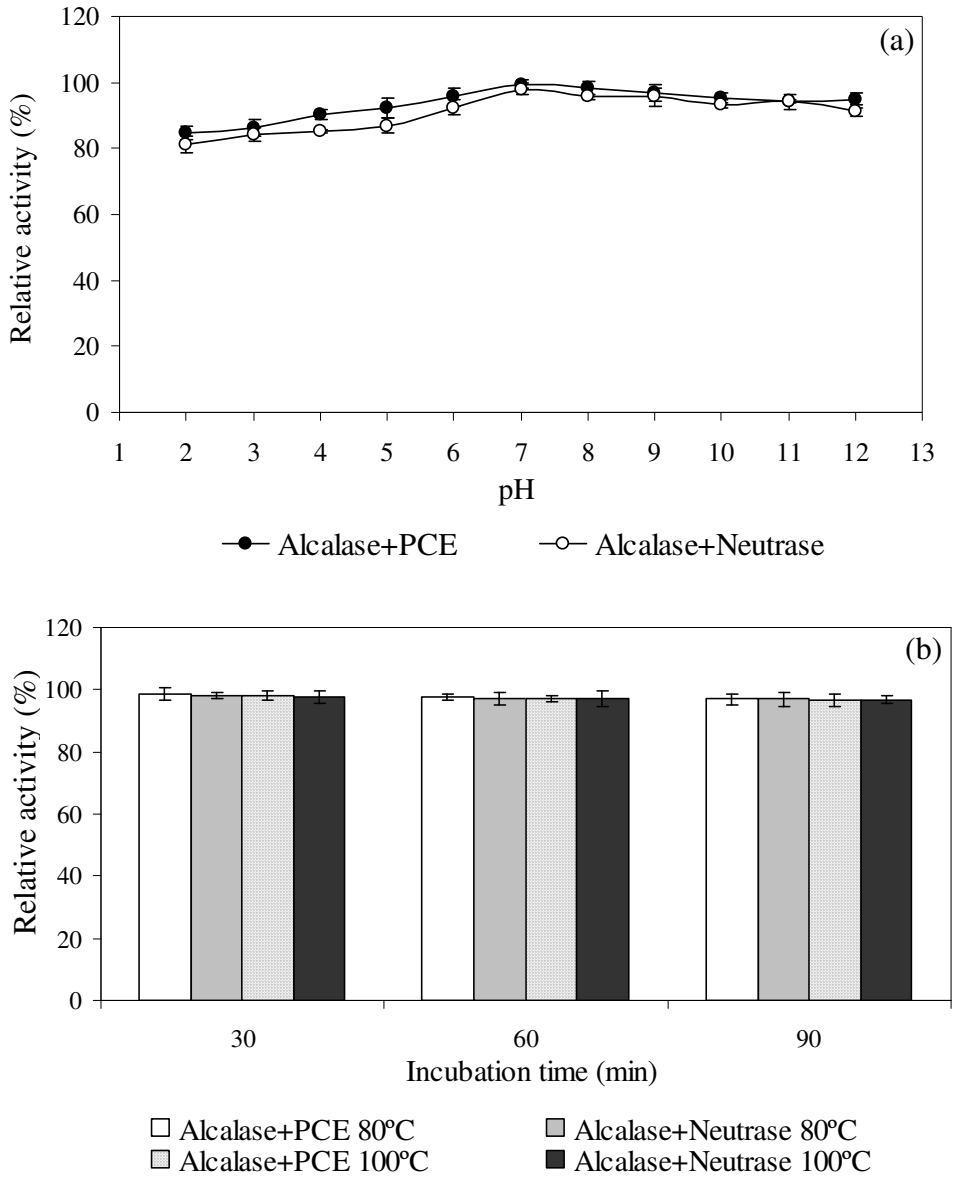

Figure 17 pH (a) and thermal (b) stabilities of antioxidative fraction from gelatin hydrolysates using Alcalase+Neutrase or Alcalase+PCE. Bars represent the standard deviation from triplicate determinations.

6. Fortification of gelatin hydrolysates in apple juice

6.1 Physical properties of apple juice fortified with gelatin hydrolysate

Physical properties (color, browning intensity and viscosity) of apple juice fortified with gelatin hydrolysates at different levels are shown in Table 8. Apple juice fortified with gelatin hydrolysates, both prepared using Alcalase+PCE and Alcalase+Neutrase had a slight decrease in L*-value and slight increase in b*-value ($p<0.05$), in comparison with the control sample. The change in L* and b*-value were more pronounced with increasing amount of hydrolysates used ($p<0.05$). At the same level of hydrolysate, apple juice fortified with gelatin hydrolysate prepared using Alcalase+Neutrase had the higher b*-value but lower L*-value than that containing hydrolysate prepared using Alcalase+PCE ($p<0.05$). Slight increase in browning intensity of apple juice was observed when both gelatin hydrolysate were added ($p<0.05$). Browning intensity increased with increasing amount of gelatin hydrolysate used ($p<0.05$). Among all treatments, apple juices, that fortified with 0.3% gelatin hydrolysate prepared using Alcalase+Neutrase showed the highest browning intensity ($p<0.05$). For the viscosity of apple juice, fortification of gelatin hydrolysates had no impact on viscosity, regardless of type and amount of hydrolysates used. In general, the fortification of both gelatin hydrolysates did not obviously change the color, browning as well as viscosity of resulting apple juice.

6.2 Chemical composition of apple juice fortified with gelatin hydrolysate

The pH, titratable acidity and hydroxyproline content of apple juice fortified with both gelatin hydrolysates at different levels are shown in Table 9. Apple juice fortified with gelatin hydrolysates had the higher pH than the control. With increasing level of gelatin hydrolysates fortified, all samples had the increases in pH ($p<0.05$). At the same level of hydrolysate fortified, the pH of apple juice with 0.3% gelatin hydrolysates prepared using Alcalase+PCE was higher than that of juice fortified with hydrolysate prepared using Alcalase+Neutrase ($p<0.05$). No difference in acidity was observed among all samples with different treatments ($p>0.05$). Acidity in apple juice is an important sensory attribute associated with its characteristic flavor and astringency (Aguilar-Rosas et al., 2007). Since the fortification of gelatin hydrolysates in apple juice did not affect acidity, the characteristic taste could be obtained. All

apple juices fortified with gelatin hydrolysates contained hydroxyproline content, while the control had no hydroxyproline. Hydroxyproline content increased with increasing gelatin hydrolysate levels used ($p<0.05$). At 0.3% gelatin hydrolysates added, the apple juice fortified with gelatin hydrolysates prepared using Alcalase+PCE showed the higher hydroxyproline content than gelatin hydrolysates prepared using Alcalase+Neutrase ($P<0.05$). Due to the different specificity in cleaving the peptide bonds in gelatin, the resulting hydrolysate might contain the different hydroxyproline contents.

Table 9 Physical properties and chemical composition of apple juice fortified with gelatin hydrolysate with different levels.

	Control (without gelatin hydrolysate)	Gelatin hydrolysate			
		Alcalase+PCE		Alcalase+Neutrase	
		0.1%	0.3%	0.1%	0.3%
Color					
L*	92.87 ± 0.01^{a†‡}	92.56 ± 0.03^{b}	92.00 ± 0.01^{c}	92.57 ± 0.01^{b}	91.93 ± 0.02^{d}
a*	-0.71 ± 0.01^{e}	-0.63 ± 0.01^{d}	-0.42 ± 0.01^{b}	-0.58 ± 0.01^{c}	-0.36 ± 0.01^{a}
b*	30.52 ± 0.04^{e}	30.68 ± 0.05^{d}	31.17 ± 0.01^{b}	30.87 ± 0.01^{c}	31.56 ± 0.03^{a}
Browning intensity (A_{420})	0.25 ± 0.00^{d}	0.26 ± 0.00^{c}	0.27 ± 0.00^{b}	0.26 ± 0.00^{c}	0.28 ± 0.00^{a}
Viscosity (cP)	0.63 ± 0.06^{a}	0.70 ± 0.1^{a}	0.73 ± 0.06^{a}	0.73 ± 0.06^{a}	0.76 ± 0.06^{a}
pH	3.76 ± 0.01^{e}	3.94 ± 0.01^{d}	4.26 ± 0.02^{a}	3.97 ± 0.00^{c}	4.23 ± 0.01^{b}
Titratable acidity (g malic acid/100 ml)	0.75 ± 0.00^{a}	0.76 ± 0.01^{a}	0.76 ± 0.01^{a}	0.76 ± 0.01^{a}	0.76 ± 0.00^{a}
Hydroxyproline (mg/L)	ND*	59.03 ± 7.32^{c}	147.92 ± 5.11^{a}	57.64 ± 5.25^{c}	130.56 ± 8.42^{b}

† Means ± SD from triplicate determinations.

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

* ND = not detected.

6.3 Antioxidantive activities of apple juice fortified with gelatin hydrolysates

Antioxidative activities of apple juice fortified with gelatin hydrolysates at different levels are depicted in Figure 18. DPPH and ABTS radical scavenging activities of apple juice fortified with both gelatin hydrolysates were higher than those of the control apple juice ($p<0.05$). The activities increased with increasing amounts of gelatin hydrolysates added ($p<0.05$). At 0.3% gelatin hydrolysates, apple juice fortified with gelatin hydrolysate prepared using Alcalase+PCE showed the higher DPPH and ABTS radical scavenging activities than apple juice fortified with gelatin hydrolysate prepared using Alcalase+Neutrase ($p<0.05$). The increases in DPPH radical scavenging activity by 24 and 19% and ABTS radicals scavenging by 28 and 22% were obtained in apple juice after fortified with gelatin hydrolysate prepared using Alcalase+PCE and Alcalase+Neutrase at a level of 3%, respectively. Nevertheless, no changes in FRAP were observed in apple juice fortified without and with both gelatin hydrolysates ($p>0.05$). From the result, apple juice showed antioxidantive activity. Apple juice has been reported as the good source of natural antioxidant such as malic acid, flavonoids, etc. (Sluis et al., 2000). The fortification of gelatin hydrolysate could improve the antioxidantive activity in apple juice, mainly from antioxidative peptides.

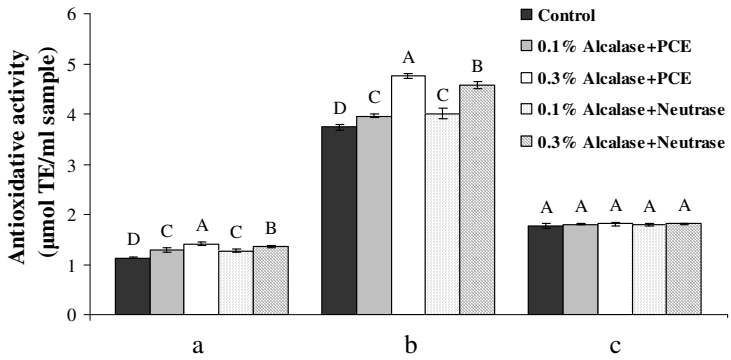

Figure 18 DPPH radical-scavenging activity (a) ABTS radical-scavenging activity (b) and ferric reducing antioxidant power (c) of apple juice fortified with gelatin hydrolysate at different levels. Bars represent the standard deviation from triplicate determinations. Different capital letters with in the same antioxidative activity assay indicate significant differences ($p<0.05$).

6.4 Sensory property of apple juice fortified with gelatin hydrolysate

Color, turbidity, odor, flavor, body and overall likeness of the control apple juice and apple juice fortified with gelatin hydrolysates at levels of 0.1 and 0.3% are shown in Table 9. The results revealed that the fortification of both gelatin hydrolysate up to 0.3% had no impact on likeness of all attributes tested of apple juice ($p>0.05$). In addition, phenol compounds are the important biochemical substances in apple juice, which combined odor–flavor characteristics in apple and apple products (Blanco et al., 2001). The polyphenols can form ionic and hydrogen bonds with amino-, hydroxyl and carboxyl groups of proteins (Fickel et al., 1999). The interaction between phenolics and macromolecules can be important factors for reducing the bitterness perception of phenolics (Pripp et al., 2004). From the result, non-significant increase in flavor likeness was noticeable in apple juice fortification with gelatin hydrolysate. Thus, gelatin hydrolysate from bigeye snapper skin could be used in fruit juice to improve sensory attributes and antioxidative activity.

Table 9 Color, turbidity, odor, flavor, body and overall likeness of apple juice fortified with gelatin hydrolysate at different levels.

Attributes	Control (without gelatin hydrolysate)[A]	Gelatin hydrolysate			
		Alcalase+PCE		Alcalase+Neutrase	
		0.1%	0.3%	0.1%	0.3%
Color	7.67 ± 0.99[a†‡]	7.63 ± 0.96[a]	7.50 ± 1.07[a]	7.57 ± 1.07[a]	7.80 ± 0.92[a]
Turbidity	7.70 ± 1.21[a]	7.87 ± 0.94[a]	7.70 ± 1.15[a]	8.00 ± 0.74[a]	8.03 ± 0.85[a]
Odor	7.38 ± 1.01[a]	7.67 ± 0.96[a]	7.33 ± 0.71[a]	7.37 ± 1.03[a]	7.32 ± 0.93[a]
Flavor	7.38 ± 1.05[a]	7.53 ± 1.07[a]	7.47 ± 1.07[a]	7.40 ± 1.04[a]	7.42 ± 1.11[a]
Body	7.20 ± 1.19[a]	7.27 ± 1.17[a]	7.40 ± 1.30[a]	7.27 ± 1.23[a]	7.27 ± 1.39[a]
Overall	7.56 ± 0.96[a]	7.62 ± 0.89[a]	7.52 ± 1.02[a]	7.33 ± 0.99[a]	7.61 ± 0.85[a]

[A] Scores were based on a 9-point hedonic scale (1: dislike extremely, 5: neither like nor dislike, 9: like extremely).

† Means ± SD from thirty determinations.

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

CHAPTER 4

CONCLUSION

1. Pyloric caeca extract (PCE) from bigeye snapper contained trypsin or trypsin-like enzyme with MW of 24 kDa. Its optimum pH and temperature were 8.0 and 55º, respectively, when casein, BAPNA and TAME were used as substrates.
2. Gelatin hydrolysates from bigeye snapper skin prepared using Alcalase, Neutrase and PCE showed the increase in antioxidative activity when DH increased. At the same DH, gelatin hydrolysate prepared using Neutrase showed the highest DPPH radical scavenging activity and ferric reducing antioxidant power (FRAP). Nevertheless, hydrolysate prepared using PCE had the highest ABTS radical scavenging activity.
3. Gelatin hydrolysates from bigeye snapper skin prepared using two-step serial hydrolysis with Alcalase and Neutrase could increase DPPH radical scavenging activity and FRAP. Serial hydrolysis with Alcalase and PCE could increase ABTS radical scavenging activity of resulting hydrolysate.
4. Gelatin hydrolysates from bigeye snapper skin prepared using Alcalase+Neutrase or Alcalase+PCE could inhibit the lipid oxidation in linoleic oxidation and lecithin liposome system in a dose-dependent manner.
5. Antioxidative peptides from bigeye snapper skin gelatin hydrolysate prepared using Alcalase+Neutrase or Alcalase+PCE was identified to have MW of 1.7 kDa. The peptides showed high pH and thermal stability and can be applied in foods processed under harsh condition.
6. Apple juice fortified with both gelatin hydrolysates prepared using Alcalase in combination with PCE or Neutrase at levels of 0.1 and 0.3% had the increase in antioxidative activity without the changes in sensory property.

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APPENDIX

ANALYTICAL METHODS

1. Lowry's procedure for quantitation of proteins (Lawry et al., 1951)

Reagents

1. A: 2% sodium carbonate in 0.1 N NaOH
2. B: 0.5% $CuSO_4.5H_2O$ in 1% sodium citrate
3. C: 2 N Folin-Ciocalteu's phenol reagent + distilled water (ratio of 1 : 1)
4. D: 50 ml reagent A + 1 ml reagent B
5. Standard reagent: Bovine serum albumin (BSA) at concentration of 1 mg/ml

Method

1. To each of eight disposable cuvette, add the following reagents according to the table.
2. Add 2 ml reagent D to each of the standards and unknown tube and then vortex immediately.
3. Incubate precisely for 10 min at room temperature.
4. Add 0.2 ml reagent C (previously dilute 1 : 1 with distilled water) and vortex immediately.
5. Incubate for 30 min at room temperature (sample incubated longer than 60 min should be discarded).
6. Read absorbance at 750 nm.
7. Plot standard curves and calculate the unknown.

Table: Experimental set up for the Lowry's assay

Tube number	Water (µl)	1 mg/ml BSA (µl)	Effective BSA Concentration (mg/ml)
1	200	0	0
2	180	20	0.1
3	160	40	0.2
4	140	60	0.3
5	100	100	0.5
6	60	140	0.7
7	0	200	1.0
8	0	0	unkhown

2. Determination of moisture content (AOAC, 2000)

Method

1. Dry the empty dish and lid in the oven at 105°C for 3 h and transfer to dessicator to cool. Weigh the empty and lid.
2. Weigh about 3 g of sample to the dish. Spread the sample to the uniformity.
3. Place the dish with sample in the oven. Dry for 3 h at 105°C.
4. After drying, transfer the dish with partially covered lid to the desiccator to cool. Reweigh the dish and its dried sample.

Calculation

$$\text{Moisture content (\%)} = \frac{(W1 - W2)}{W1} \times 100$$

where W1 = weight (g) of sample before drying

W2 = weight (g) of sample after drying

3. Determination of protein content (AOAC, 2000)

Reagents

1. Kjedahl catalyst: Mix 9 part of potassium sulphate (K_2SO_4) with 1 part of copper sulphate ($CuSO_4$)
2. Sulfuric acid (H_2SO_4)

3. 40% NaOH solution (w/v)
4. 0.2 N HCl solution
5. 4% H_3BO_3 solution (w/v)
6. Indicator solution: Mix 100 ml of 0.1% methyl red (in 95% ethanol) with 200 ml of 0.2% bromocresol green (in 95% ethanol)

Method

1. Place sample (0.5-1.0 g) in digestion flask.
2. Add 5 g Kjedahl catalyst and 20 ml of conc. H_2SO_4
3. Prepare a tube containing the above chemical except sample as blank. Place flasks in inclined position and heat gently unit frothing ceases. Boil briskly until solution clears.
4. Cool and add 60 ml distilled water cautiously.
5. Immediately connect flask to digestion bulb on condenser and with tip of condenser immersed in standard acid and 5-7 indicator in receiver. Rotate flask to mix content thoroughly; then heat until all NH_3 is distilled.
6. Remove receiver, wash tip of condenser and titrate excess standard acid distilled with standard NaOH solution.

Calculation

$$\text{Protein content (\%)} = \frac{(A-B) \times N \times 1.4007 \times 6.25}{W}$$

where A = volume (ml) of 0.2 N HCl used sample titration
B = volume (ml) of 0.2 N HCl used in blank titration
N = normality of HCl
W = weight (g) of sample
14.007 = atomic weight of nitrogen
6.25 = the protein-nitrogen conversion factor for fish and its by-products

4. Determination of ash content (AOAC, 2000)

Method

1. Place the crucible and lid in the furnace at 550°C overnight to ensure that impurities on the surface of crucible are burned off.
2. Cool the crucible in the desiccator (30 min).
3. Weigh the crucible and lid to 3 decimal places.
4. Weigh about 5 g sample into the crucible. Heat over low Bunsen flame with lid half covered. When fumes are no longer produced, place crucible and lid in furnace.
5. Heat at 550°C overnight. During heating, do not cover the lid. Place the lid after complete heating to prevent loss of fluffy ash. Cool down in the desiccator.
6. Weigh the ash with crucible and lid when the sample turns to gray. If not, return the crucible and lid to the furnace for the further ashing.

Calculation

$$\text{Ash content (\%)} = \frac{\text{Weight of ash}}{\text{Weight of sample}} \times 100$$

5. Determination of fat content (AOAC, 2000)

Reagent

1. Petroleum ether

Method

1. Place the bottle and lid in the incubator at 105^{o}C overnight to ensure that weight of bottle is stable.
2. Weigh about 3-5 g of sample to paper filter and wrap.
3. Take the sample into extraction thimble and transfer into soxhlet.
4. Fill petroleum ether about 250 ml into the bottle and take it on the heating mantle.
5. Connect the soxhlet apparatus and turn on the water to cool them and then switch on the heating mantle.
6. Heat the sample about 14 h (heat rate of 150 drop/min).

7. Evaporate the solvent by using the vacuum condenser.
8. Incubate the bottle at 80-90^{o}C until solvent is completely evaporated and bottle is completely dried.
9. After drying, transfer the bottle with partially covered lid to the desiccator to cool. Reweigh the bottle and its dried content.

Calculation

$$\text{Fat content (\%)} = \frac{\text{Weight of fat}}{\text{Weight of sample}} \times 100$$

6. Determination of hydroxyproline content (Bergman and Loxley, 1963)

Reagents

1. 6 N HCl
2. Oxidant solution (the mixture of 7% (w/v) chlororamine T and acetate/citrate buffer, pH 6 at a ratio of 1:4 (v/v))
3. Ehlich's reagent solution (the mixture of solution A (2g of *p*-dimethylamino benzaldehyde in 3 ml of 60% (v/v) perchloric acid (w/v))
4. Isopropanol
5. Hydroxyproline standard solution (400 ppm)

Method

Sample preparation:

1. Weigh about 0.1-2.0 g sample (depending on type of sample) into screw cap tube.
2. Add 6 N HCl into the sample at the ratio of 1 : 10 (solid/acid, w/v).
3. Heat at 110°C for 24 h in oil bath.
4. Clarify the hydrolysate with activated carbon and filter through Whatman No. 4 filter paper.
5. Neutralize the filtrate with 10 M NaOH and 1 M NaOH to obtain the pH 6.0-6.5.

Hydroxyproline determination:

1. Transfer 0.1 ml of the neutralized sample into a test tube and add 0.2 ml of isopropanol then mix well.
2. Add 0.1 ml of oxidant solution and mix well.

3. Add 1.3 ml of Ehrlich's reagent solution.
4. Heat the mixture at 60°C for 25 h in the water bath and then cool for 2-3 min in running water.
5. Add isopropanol at ratio of 3 : 13 (mixture/isopropanol, v/v) and mix well.
6. Read absorbance at 558 nm.
7. Plot the standard curves and calculate the unknown.

Table: Experimental set up for the hydroxyproline's assay

Tube number	Water(µl)	400 ppm Hydroxyproline (µl)	Effective Hydroxyproline Concentration (ppm)
1	100.0	0.0	0
2	97.5	2.5	10
3	95.0	5.0	20
4	92.5	7.5	30
5	90.0	10.0	40
6	87.5	12.5	50
7	85.0	15.0	60
8	0.0	0.0	unknown

7. Biuret method for quantitation of protein (Robinson and Hodgen, 1940)

Reagents

1. Biuret reagent: combine 1.50 g $CuSO_4.5H_2O$, 6.00 g sodium potassium tartrate, and 500 ml distilled water in a beaker and stir, add while stirring 300 ml of 10% NaOH (w/v), transfer to plastic bottle for storage.
2. Distilled water
3. Standard reagent: 10 mg/ml bovine serum albumin (BSA)

Method

1. To each of seven disposable cuvette, add the following reagents according to the table.
2. Add 2.0 ml of the biuret reagent to each tube, and mix well.
3. Incubate the mixture at room temperature for 30-45 min, and then read the adsorbance of each tube at 540 nm.

5. For tube 1-5, plot the absorbance at 540 nm as a function of effective BSA concentration and calculate the best fit straight line from data. Then, use the average absorbance for the three sample of unknown and read the concentration of sample from the plot.

Table: Experimental set up for the Biuret's assay

Tube number	Water (µl)	10 mg/ml BSA (µl)	Effective BSA Concentration (mg/ml)
1	500	0	0
2	400	100	2
3	300	200	4
4	200	300	6
5	100	400	8
6	0	500	10
7	0	0	unknown

8. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) (Laemmli, 1970)

Reagent

1. 30% Arylamide-0.8% bis Acrylamide
2. Sample buffer: Mix 30 ml of 10% of SDS, 10 ml of glycerol, 5 ml of β-mercaptoethanol, 12.5 ml of 50 mM Tris-HCl, pH 6.8, and 10 mg bromophenol blue. Bring the volume to 100 ml with distilled water. Divide into 1 ml aliquots, and store at -20°C.
4. 2% (w/v) Ammonium persulfate
5. 1% (w/v) SDS
6. TEMED (*N,N,N'N'*- tetramethylethylenediamine)
7. 0.5 M Tris-HCl, pH 6.8
8. 1.5 M Tris-HCl, pH 8.8
9. Electrode buffer: Dissolve 3 g of Tris-HCl, 14.4 g of glycine and 1 g of SDS in distilled water. Adjust to pH 8.3 and being the volume to 1 liter using distilled water.

10. Staining solution: Dissolve 0.05 g of Coomassie blue R-250 in 15 ml methanol. Add 5 ml of glacial acetic and 80 ml of distilled water.
11. Destaining solution: 30% methanol-10% glacial acetic acid

Method

Pouring the running gel:

1. Assemble the minigel apparatus according to the manufacture's detailed instructions. Make sure that the glass and other components are rigorously clean and dry before assembly.
2. Mix the separating gel solution by adding as defined in following Table.
3. Transfer the separating gel solution using a Pasture pipettes to the center of sandwich is about 1.5 to 2 cm from the top of the shorter (front) glass plate.
4. Cover the top of the gel with a layer of distilled water by squiting the distilled water against the edge of one of the spacers. Allow the resolving gel to polymerize fully (usually 30-60 min).

Pouring the stacking gel:

1. Pour off completely the layer of isobutyl alcohol.
2. Prepare a 4% stacking gel solution by adding as defined in Table.
3. Transfer stacking gel solution to tickle into the center of the sandwich along an edge of the one of the spacers.
4. Insert comb into the layer of stacking gel solution by placing one corner of the comb into the gel and slowly lowering the other corner in. Allow the attacking gel solution to polymerize 30 to 45 min at room temperature.

Table: Experimental set up for running and stacking gel

Reagents	7.5% running gel	4% stacking gel
30% Acrylamide-bis	2.500 ml	0.665 ml
1.5 M Tria-HCl buffer, pH 8.8	2.500 ml	-
0.5 M Tris-HCl buffer, pH 6.8	-	1.25 ml
Distilled water	4.845 ml	3.00 ml
10% SDS	10 µl	50 µl
2% Ammonium persulfate	50 µl	25 µl
TEMED	5 µl	3 µl

Sample preparation:

1. Weigh 3 g of gelatin and completely dissolve with distilled water in a final volume of 30 ml.
2. Centrifuge at 3,500 x g for 5 min at ambient temperature and collect supernatant.

Loading the gel:

1. Dilute the protein to be 4:1 (v/v) with sample buffer in microcentrifuge tube and boil for 1 min at 100°.
2. Remove the comb without tearing the edge of the polyacrylamide wells.
3. Fill the wells with electrode buffer.
4. Place the upper chamber over the sandwich and lock the upper buffer chamber to the sandwich. Pour electrode buffer into the lower buffer chamber. Place the sandwich attached to the upper buffer chamber into the lower chamber.
5. Fill the upper buffer chamber with electrode buffer so that the sample wells of the stacking gel are filled with buffer.
6. Use a 10-25 µl syringe with a flate-tipped needle; load the protein sample into the wells by carefully applying the sample as a thin layer at the bottom of the well.
7. Fill the remainder of the upper buffer chamber with additional electrode buffer.

Running the gel:

1. Connect the power supply to the anode and cathode of the gel apparatus and run at 50 V and 150 V.
2. After the bromophenol blue tracking dye has reached the bottom of the separating gel, disconnect the power supply.

Disassembling the gel:

1. Remove the upper buffer chamber and the attached sandwich.
2. Orient the gel so that the order of the sample well is known, remove the sandwich from the upper buffer chamber, and lay the sandwich on a sheet of absorbent paper or paper towels. Carefully slide the spacers out from the edge of the sandwich along its entire length.
3. Insert a spatula between the glass plates at one corner where the spacer was and gently pry the two plates apart.
4. Remove the gel from the lower plate. Place the plate with the gel attached into the shallow dish of fixing agent or dye and swishing the plate.

Staining the gel:

1. Plate the gel in a small plastic box and cover with the staining solution. Agitate slowly for 3 h. or more on a rotary rocker.
2. Pour off the staining solution and cover the gel with a solution of destaining solution I. Agitate slowly for about 15 min.
3. Pour off the destaining solution I and replace with flesh solution. Repeat

until the gel is clear except for the protein bands

9. Measurement of ferric thiocyanate method (Thiansilakul et al., 2007)

Reagent

1. 75% ethanol
2. 30% ammonium thiocyanate
3. 20 mM ferrour chloride solution in 3.5% HCl

Method

1. To 50 ml of the mixture, add 2.35 ml of 75% ethanol, 50 ml of 30% ammonium thiocyanate and 50 ml of 20 mM ferrous chloride solution in 3.5% HCl.

2. Mix the mixture.
3. Read the absorbance of the colored solution at 500 nm after 3 min.

10. Measurement of TBARS (Lee and Hendricks, 1997)

Reagent

1. TBA solution (15% TCA/ 0.375% TBA/ 0.025N HCl)
2. 0.2 % BHT

Method

1. Mix liposome sample (1 ml) with 20 µl of butylated hydroxytoluene (0.2%) and add 2 ml of TBA solution into the mixture.
2. Heat the mixtures for 10 min in a boiling water bath (95-100^{o}C) to develop pink color.
3. Cool with tap water and centrifuge for 20 min at 5,500 × g.
4. Read the absorbance of the supernatant containing the pink chromogen at 532 nm and express TBARS as mg MDA/ml liposome.

11. Measurement of conjugated diene (Frankel et al., 1997)

1. Dissolve liposome samples (0.1 ml) in methanol (5.0 ml).
2. Read the absorbance at 234 nm.

12. Titratable acidity (AOAC, 2000)

Reagent

1. 1% phenolphthalein solution
2. 0.1 N NaOH

Method

1. Added 10 ml of sample in 250 ml volumetric containing 50 ml of distilled water.
2. Added 2-3 drops of 1% phenolphthalein solution.
3. Titrate with 0.1 N NaOH until pink color (end-point) appears.
4. Record the volume of NaOH used.

Calculation

$$\text{Titratable acidity (g malic acid/100 ml)} = \frac{0.1 \times \text{volume of NaOH} \times 0.134 \times 100}{\text{Volume of sample (ml)}}$$

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