CHAPTER 3

RESULTS AND DISCUSSION

1. Extraction and characterization of pepsin from bigeye snapper stomach

1.1 Extraction and fractionation of pepsin from bigeye snapper stomach

Effect of extracting media on pepsin extraction from bigeye snapper stomach is shown in Table 3. Stomach extract using 50 mM Na-phosphate, pH 7.2 showed the higher proteinase activity than those extracted with distilled water and 50 mM Tris-HCl buffer when hemoglobin was used as a substrate. Additionally, specific activity was greater in the extract using Na-phosphate buffer, compared with other two extracts. The results suggested that Na-phosphate buffer had a greater ability to extract pepsin with the higher purity than did Tris-HCl buffer. Phosphate in the buffer might favor the solubilization of pepsin associated with the cell membrane by increasing the charge of enzymes and proteins. The repulsion between the enzymes and stomach tissues might lead to the ease of pepsin extraction from the stomach tissues. Generally, the buffer is needed to protect the enzymes from large quantities of acids released from the vacuoles on rupture of the cell (Whitaker, 1994). During assay at low pH (pH 2.5), pepsin could be activated. Inactive pepsinogen, which is produced by chief cell of stomach wall and quite stable at pH 7-9, was rapidly converted to active pepsin at low pH (Al-Janabi *et al.*, 1972; Whitaker, 1994). Bohak (1973) found that the chicken pepsinogen undergoes a rapid conformational change to form the intermediate, and the subsequent conversion of this intermediate to the active enzyme take place in pH range of 2-4. Al-Janabi *et al.* (1972) observed the spontaneous and pepsin-catalyzed activation of porcine pepsinogen at pH 4 with a predominantly intermolecular activation and at pH below 3 with intramolecular activation. From the result, buffers could maintain the pH during the extracton at pH 7.2. As a result, pepsinogen was still the predominant form and was not able to cause autolysis. With the extracting method using water, pepsinogen might be activated to pepsin induced by acid released. Thus, pepsin, an inactive form, could be autolyzed, leading to the loss in activity.

Table 3 Effect of extracting media on pepsin extraction*

Extracting media	Stomach tissue wt. (g)	Total protein (mg)	Total activity (Units)	Specific activity** (U/mg protein)	Proteinase activity** (U/g tissue)
Distilled water	3.01	60.9	50,601	831.4±24.4b	16,838.3±494.7a
50 mM Na-phosphate, pH 7.2	3.00	74.5	61,592	829.2±73.3b	20,576.35±1,819.2b
50 mM Tris-HCl, pH 7.2	3.01	105.6	55,945	528.7±24.5a	18,555.4±859.4a

Mean±S.D. from triplicate determinations.

*The proteinase activity was measured using hemoglobin as substrate. The assay was carried out at pH 2.5 and 45^{o}C.

**The different letters in the same column denote the significant differences ($P<0.05$).

Fractionation of pepsin from bigeye snapper stomach using different precipitation methods is summarized in Table 4. Ammonium sulfate and acetone were used for pepsin precipitation. Fractionation using ammonium sulfate with 0-20% saturation resulted in the highest proteinase recovery with the yield of 73.1%. A large amount of contaminating proteins were removed with small loss in enzyme activity, leading to an increase in purity by 33.7-fold. Some pepsin activity was found in the fraction obtained from ammonium sulfate precipitation at 20-40% saturation. Nevertheless, no pepsin was obtained in other fractions. Ammonium sulfate is used for protein precipitation at pHs below 5.5. Protonation of many carboxylate groups in proteins make them become increasingly positive charged. As a consequence, the formation of protein-sulfate complexes more likely occurs (Scopes, 1978). Those proteins tend to precipitate out at lower salt concentration (0-20% saturation). However, large amounts of contaminating proteins were still remained after precipitating up to 40% saturation. Those proteins were mostly precipitated when ammonium sulfate at levels greater than 40% saturation was used.

Table 4 Fractionation of pepsin from bigeye snapper stomach*

Fractionation	Total activity** (units)	Total protein (mg)	Specific activity (units/mg)	Purity (fold)	Yield (%)
Ammonium sulfate					
Crude extract	63,947	157.5	406.0	1	100
0-20% Saturation	46,753	3.4	13,674.5	33.7	73.1
20-40% Saturation	11,553	7.0	1,641.5	4.0	18.1
40-60% Saturation	0	14.2	0	0	0
60-80% Saturation	0	11.6	0	0	0
Residue	0	18.0	0	0	0
Acetone					
Crude extract	73,566	170.5	431.5	1	100
0-20% v/v	549	3.9	141.1	0.3	0.7
20-40% v/v	70	7.2	9.6	0.02	0.1
40-60% v/v	0	10.1	0	0	0
60-80% v/v	27,374	13.9	1,963.7	4.5	37.2
Residue	0	132.3	0	0	0

*The proteinase activity was measured using hemoglobin as substrate. The assay was carried out at pH 2.5 and 45°C.

**The unit of enzyme activity is expressed as nmoles of tyrosine liberated per min.

For acetone precipitation, the highest purity and yield were found in the presence of 60-80% acetone. The purification fold of 4.5 with a yield of 37.2% was obtained (Table 4). When comparing both precipitation methods, pepsin fraction from acetone (60-80%) precipitation showed the lower purity and yield than ammonium sulfate (0-20% saturation) fraction. This was postulated that acetone might cause the denaturation of pepsin, resulting in the decreased activity. Scope (1978) reported that the most proteins loss their stability in the presence of organic solvents. All organic solvents have a tendency to denature proteins, especially at temperature above 0°C, since all hydrophobic force within molecules is weakened by the less polar solvent

(Scope, 1978; Englard and Seifter, 1990). From the result, the activity loss of 62% was found when acetone precipitation was used, possibly caused by instability of pepsin in the presence of this solvent.

1.2 pH and temperature profile of bigeye snapper pepsin (BSP)

The effects of pH on the proteolytic activity of partially purified proteinase from stomach extract referred to as 'bigeye snapper pepsin; BSP' are shown in Figure 5. The optimal pH for hydrolysis of hemoglobin of BSP was found at pH 2.5. Therefore, this proteinase was considered to be an acid proteinase, most likely pepsin. The optimal pH of BSP was similar to those of pepsin obtained from stomach or gastric mucosa of Pacific yellowfin tuna (Norris and Mathies, 1953), Atlantic cod (Brewer *et al.*, 1984), dogfish (Guerard and Le Gal, 1987) and North Pacific bluefin tuna (Tanji *et al.*, 1988) when hemoglobin was used as a substrate. The optimal pH was determined by the substrate used. When milk casein was used as a substrate, the optimal pH of hog pepsin and bonito pepsin were 1.6 and 2.0, respectively. However, the optimal pH was changed to 2.0 for hog pepsin and slightly more than 2 for bonito pepsin when hemoglobin was used as a substrate (Kubota and Ohnuma, 1970). The optimal pH of Greenland cod protease I was 3.5 and 3.0 when hemoglobin and casein were used as the substrate, respectively (Squires *et al.*, 1986). Moreover, activity of pepsin was changed drastically as the substrate concentrations varied (Gildberg *et al.*, 1990). Specific activity at pH 3 of pepsin I from Atlantic cod stomach increased 3.5 times when hemoglobin concentration increased from 1 to 4%, whereas the pH optima changed from 3.7 to 3.0 (Gildberg *et al.*, 1990). Therefore, the differences in optimal pH were attributed to the accessibility of the substrate to the active site at the particular pH environment (Mason and Barett, 1984). The activities of BSP slightly decreased at very acidic pH and sharply decrease as the pH increased up to 5. No activity was found at pHs 6 and 7. At pHs higher and lower than the optimal pH, the enzyme possibly underwent the conformational changes, cause by charge repulsion. These caused the proteins to unfold in order to decrease the electrostatic bond and minimize the free energy change (Benjakul *et al.*, 2003a; Campos and Sancho, 2003). From the result, the complete losses in proteolytic activity of BSP were observed at above 6. The result was in accordance with the phenomenon found in most pepsin from other species (Gildberg, 1988). Twining *et al.* (1983) found that the cognate pepsin from rainbow trout was denatured at pH value above 7.

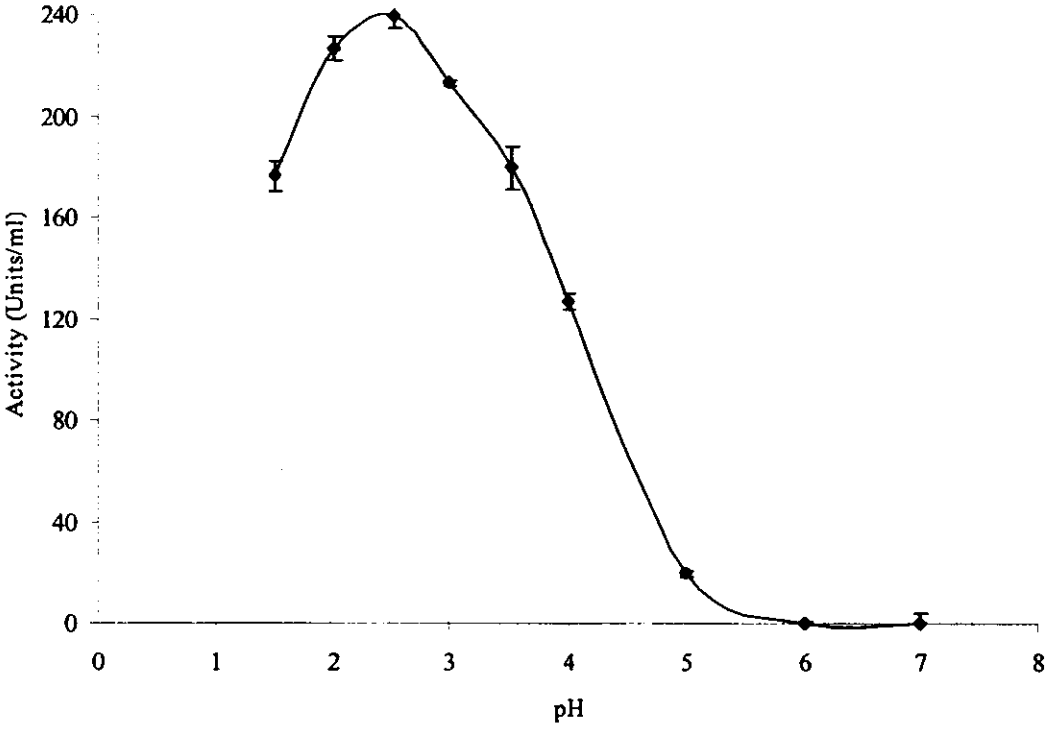

Figure 5 pH profiles of proteinases from bigeye snapper stomach. Proteinase activity was determined using hemoglobin as a substrate at 45°C at various pHs for 20 min. TCA-soluble peptides released were determined by the Lowry assay. The activity was expressed as units/ml. Bars represent the standard deviation from triplicate determinations.

Temperature profile of BSP is depicted in Figure 6. The optimal temperature of BSP was noticeable at 45°C when hemoglobin was used as a substrate. The optimal temperature of BSP was similar to those of pepsin obtained from dogfish (Guerard and Le Gal, 1987), Arctic capelin, North Sea herring and Indian oil sardine (Gildberg, 1988). Yellowfin tuna pepsin (Norris and Mathies, 1953) and purified capelin pepsin (Gilgberg and Raa, 1983) had the optimal temperature at 42°C and 40°C, respectively. The differences in optimal temperature might be associated with the differences in enzyme conformation as influenced by habitat, environment and genetics (Gildberg, 1988). A sharp decrease in activity was observed at temperature above 50°C, possibly due to thermal denaturation of enzyme. No activity was found at 70°C, suggesting the complete denaturation of enzyme. Thermal denaturation of proteins or enzymes

might be associated by the conformational changes or aggregation of those proteins or enzymes induced at high temperature (Tello-Solís and Romero-García, 2001).

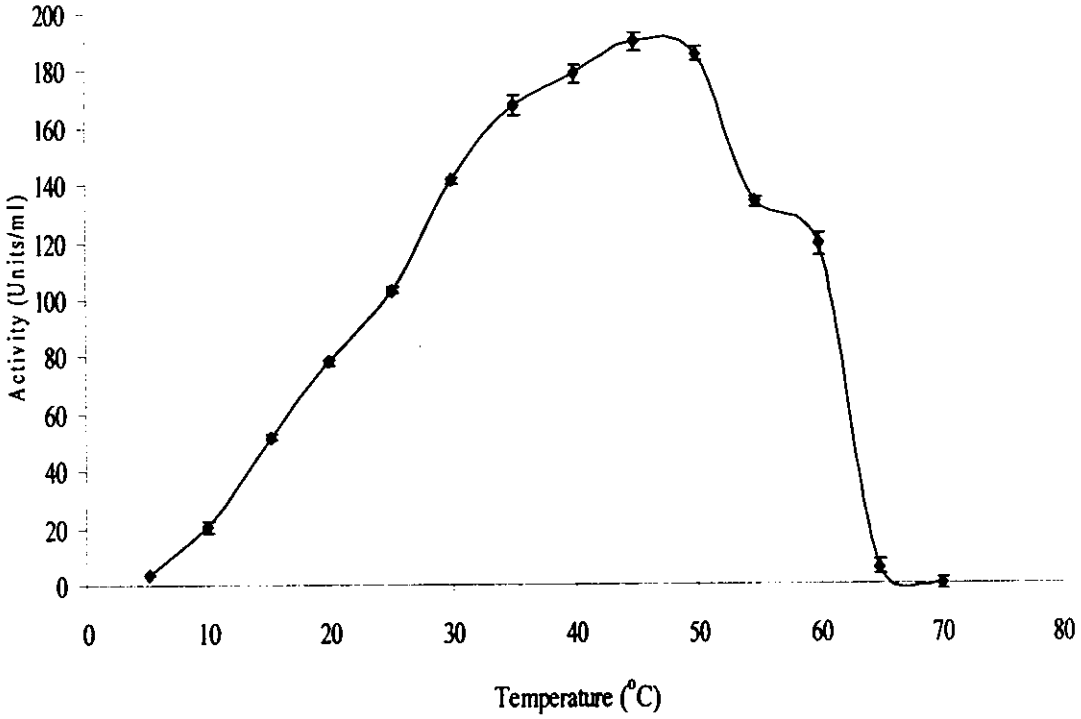

Figure 6 Temperature profiles of proteinases from bigeye snapper stomach. Proteinase activity was determined using hemoglobin as a substrate at pH 2.5 at various temperatures for 20 min. TCA-soluble peptides released were determined by the Lowry assay and the activity was expressed as units/ml. Bars represent the standard deviation from triplicate determination.

1.3 pH and thermal stability of BSP

pH stability of BSP is shown in Figure 7. BSP was stable in the pH range of 1-6 with the exposure time of 30-120 min, in which residual activity more than 80% was found. With an extended incubation time, proteinase activity was lost to a greater extent, possibly due to autolysis of pepsin, particularly at pH 2.0. The marked decrease in activity was noticeable at pH above 6. Similar results were reported for Monterey acidic enzymes (Castillo-Yanez *et al.*, 2004), sardine acidic proteinase (Noda and Murakami, 1981) and yellowfin tuna pepsin (Norris and Mathies, 1953). Those enzymes were stable at pH range of 2-6 and became susceptible to activity

losses at neutral and alkaline pH. Gildberg (1988) reviewed that pepsins from cold and temperate water fishes have the stability in the pH range of 2-5, whereas pepsins from warm water species such as bonito are quite stable even in neutral conditions (Kubota and Ohnuma, 1970). However, fish pepsin was reported to renaturize when incubated in acidic pH (Diaz-Lopez *et al.*, 1998).

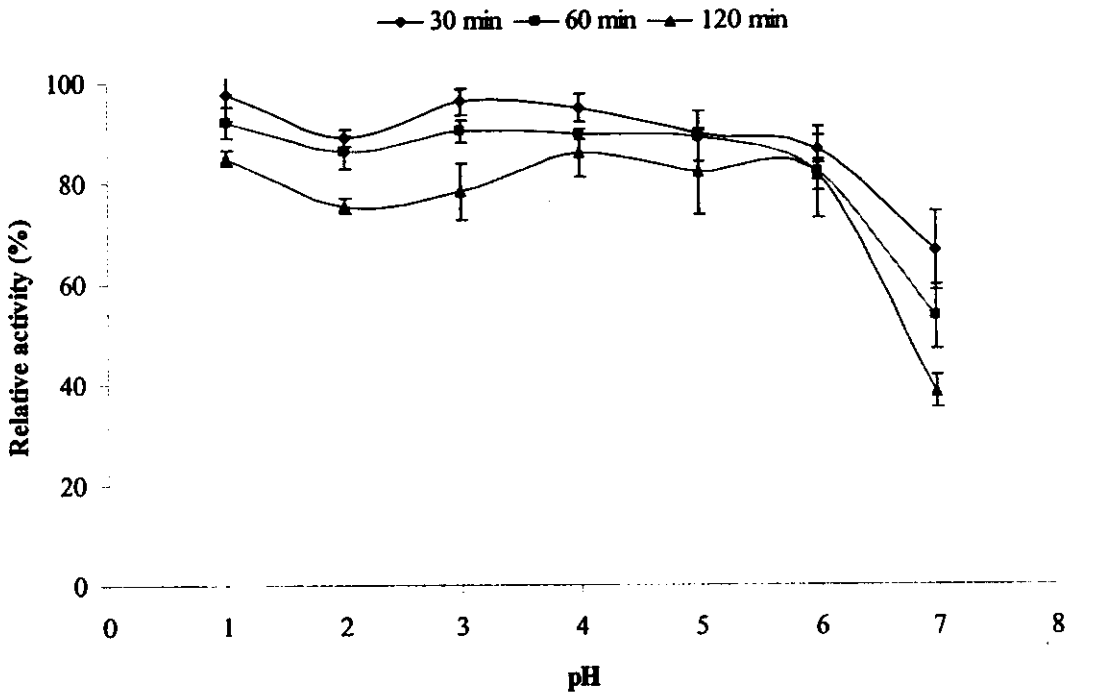

Figure 7 pH stability of proteinases from bigeye snapper stomach. Enzyme at a level of 184.84 units/ml was subjected to different pHs for 30, 60 and 120 min. Residual activity was determined at 45°C and pH 2.5 for 20 min using hemoglobin as a substrate. Bars represent the standard deviation from triplicate determinations.

Thermal stability of BSP is shown in Figure 8. BSP was stable when incubated at temperature up to 40°C for 30-120 min. Nevertheless, the sharp decrease in activity was noticeable at temperature above 50°C. No activity was remained at 60°C, suggesting that complete loss in activity caused by thermal denaturation of proteinases, mostly pepsin. The thermal stability of BSP was similar to that reported for dogfish pepsin, which was stable at temperature below 50°C (Guerard and Le Gal, 1987). At high temperature, enzyme possibly underwent denaturation and lost its activity. Fish pepsins showed the low thermostability but

exhibited high activity at low temperature for cold water species (Arunchalam and Haard, 1985; Gilgberg *et al.*, 1990). Additionally, fish pepsins contain more basic amino acids in the polypeptide chain than mammalian pepsins (Haard, 1994). Potential number of disulfide linkages, the average hydrophobicity and the amount of intramolecular hydrogen bonds are considered to affect thermostability of enzymes (Gildberg *et al.*, 1990). Tello-Solís and Romero-García (2001) observed the aggregation of porcine pepsin at temperature higher than 50 °C. Therefore, the irreversibility of pepsin may be associated with aggregation and kinetic factors.

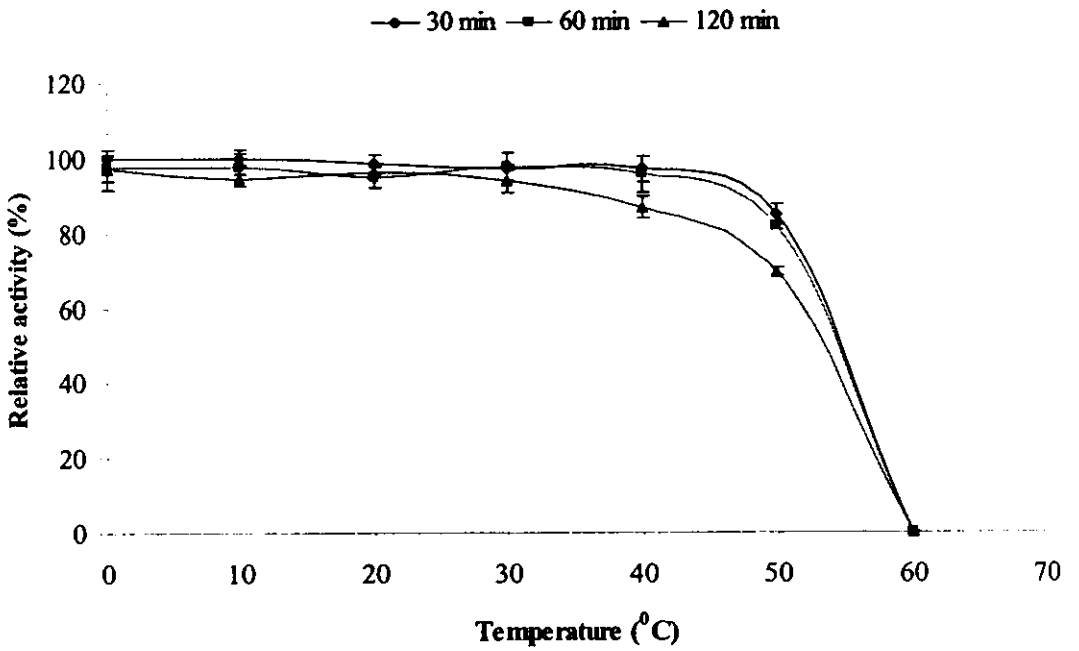

Figure 8 Thermal stability of proteinases from bigeye snapper stomach. Enzyme at a level of 181.15 units/ml was subjected to incubation at different temperatures for 30, 60 and 120 min, followed by cooling in iced water. Residual activity was determined at 45 °C and pH 2.5 for 20 min using hemoglobin as a substrate. Bars represent the standard deviation from triplicate determinations.

1.4 Effect of inhibitors on BSP

The effect of the various inhibitors on proteinase activity of BSP was determined as shown in Table 5. The proteolytic activity was strongly inhibited by pepstatin A with the range of 1-100 μM. Nevertheless, EDTA, soybean trypsin inhibitor and E-64 showed the negligible

inhibitory effect. Pepstatin A inhibits most aspartic proteinase (Umezawa, 1976) with the exception of rennin and retroviral aspartic proteinases (Fusek and Větvička, 1995). Fersht (1984) proposed the statine residue has a tetrahedral carbon replacing the normal carboxyl carbon of aspartic proteinase, resulting in the loss in proteinase activity. Fujimoto *et al.* (2004) proposed that the crystal structure of aspartic proteinase from *Irpex lacteus* formed the complex with pepstatin, leading to the transition state of the catalytic mechanism. Pepstatin, a pentapeptide, is very specific inhibitor to forms multiple, noncovalent interactions with the active site of aspartic proteinases and blocks the accessibility of a substrate to the active site cleft (Hard, 1994; Fusek and Větvička, 1995). Guerard and Le Gal (1987) reported that the activity of dogfish pepsin II was progressively reduced to about 50% of its initial value when pepstatin A at a level of 6×10^{-7} M was added, while a total inhibitory effect was obtained at 10^{-6} M. From the result, the major proteinase in bigeye snapper extract was aspartic proteinase, most likely pepsin.

Table 5 Effect of various inhibitors on the activity of proteinases from bigeye snapper stomach

Inhibitors	Concentration	% inhibition*
PepstatinA	100μM	100
	10μM	100
	1μM	100
EDTA	2mM	2.22±0.25
Soybean trypsin inhibitor	0.1mM	3.20±0.13
E-64	0.1mM	4.17±0.75

*Mean±SD from triplicate determinations. Residual activity was analyzed using hemoglobin as a substrate at 45°C and pH 2.5 for 20 min.

2. Preparation and characterization of pepsin-solubilized collagen from bigeye snapper skin

2.1 Effect of pepsin on collagen extraction and composition

2.1.1 Effect of pepsin levels and reaction time

The yield of collagen extracted from bigeye snapper skin using BSP and PP for 24 and 48 h is shown in Table 6. The highest yield (53.68%) was obtained when the skin was extracted using BSP at 20 kUnits/g defatted skin for 48 h. At the same enzyme level and reaction time, BSP showed the higher extraction efficiency than did PP ($P<0.05$) as evidenced by the greater yield. High enzyme level generally led to the greater yield of collagen extracted. Additionally, the longer reaction time rendered the higher yield. Hydrolysis at telopeptide region was more pronounced with the sufficient reaction time. particularly at the higher enzyme levels. From the result, a much lower yield was obtained in the absence of pepsins both BSP and PP. The collagen extracted was mainly acid-solubilized collagen, which was more solubilized with increasing extraction times. The electrophoretic patterns of resultant collagen under reducing condition are shown in Figure 9. All collagens extracted under different conditions had the similar components. β-chain was found as the dominant constituent. α-chains, both α1 and α2, were also observed in all collagens extracted. From the protein pattern, it was revealed that collagen obtained was type I collagen, which was predominant in fish skin collagen (Ogawa *et al.*, 2003; Jongjareonrak *et al.*, 2005b; Kittipattanabawon *et al.*, 2005; Hwang *et al.*, 2007). The result suggested that the covalent cross-linking at the telopeptide regions of collagen molecules through the condensation of aldehyde groups as well as the inter-molecular cross-linked molecules were not readily solubilized by acid extraction (Jongjareonrak *et al.*, 2005b). These cross-linking molecules generally caused the decreased in solubility of collagen (Foegeding *et al.*, 1996; Burghagen, 1999). Cross-linked molecules at telopeptide region were cleaved by pepsin, both BSP and PP, without damaging the integrity of triple helix of collagen. With increasing extraction time, collagens referred to as 'pepsin-solubilized collagen' localized in the loosen structure caused by partial cleavage at telopeptide region, could be more extracted. From the result, the higher band intensity of β- and α-chains was noticeable in pepsin-solubilized collagen using BSP, compared with that of collagen extracted using PP as the extraction aid. Molecular weight of α1 and α2 of pepsin-solubilized collagens were slightly decreased when increasing extraction time (Figure 9). Furthermore, slightly lower molecular weight was obtained in β- and α-chains of

pepsin-solubilized collagen, compared with acid-solubilized collagen (without pepsin addition). The result suggested that small portions of peptides at telopeptide region were cleaved and removed, leading the formation of collagens with slightly lower MW.

Table 6 Total hydroxyproline (Hyp) and yield of collagen extracted from bigeye snapper skin using bigeye snapper pepsin (BSP) or porcine pepsin (PP) for 24 h and 48 h

Extraction time	Treatment	Total extracted Hyp (mg/g defatted skin)	Yield** (%)
24 h	Control	1.35	5.67±0.39a***
	BSP 10*	6.86	28.79±1.37e
	BSP 20	8.08	33.90±0.71f
	PP 10	4.79	20.11±1.05c
	PP 20	5.30	22.24±1.07d
48 h	Control	1.78	7.49±2.36b
	BSP 10	12.20	51.18±0.40h
	BSP 20	12.79	53.68±0.71i
	PP 10	8.06	33.82±0.40f
	PP 20	10.43	43.78±0.41g

Mean±SD from triplicate determinations.

*Numbers denote activity of enzyme in kUnits/g defatted skin.

**Yield was calculated based on hydroxyproline content in the collagen in comparison with that of the skin.

***Different letters in the same column indicate the significant difference ($p<0.05$).

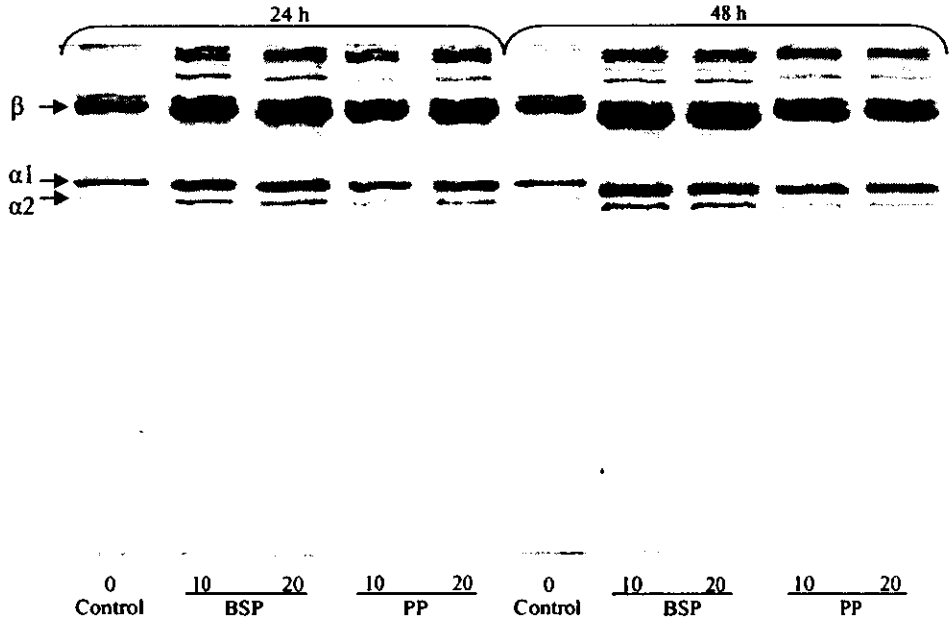

Figure 9 SDS-PAGE patterns of collagens from bigeye snapper skin extracted using BSP or PP for 24 h and 48 h. Numbers denote pepsin activity in kUnits/g defatted skin.

2.1.2 Effect of prior acid swelling process in combination with pepsin

The collagen extraction was maximized with pre-swelling process. The skin was swollen with 0.5 M acetic acid for 24 h (A24) and 48 h (A48) prior to the hydrolysis using BSP or PP for 24 or 48 h. Total hydroxyproline and the yield of all treatments are shown in Table 7. The yield of collagen extracted increased with increasing acid swelling time and pepsin treatment time. The highest yield (~65%) was found when BSP was added with the reaction time of 48 h regardless of acid swelling time. The results suggested that swollen skin, which was treated with acetic acid, possibly had a porous and loose structure by the charge repulsion. As a result, the penetration of pepsin into the skin matrix could be enhanced. Thus, hydrolytic reaction of pepsin toward collagen was augmented. From the result, BSP had the greater ability to extract or solubilize the collagen from the skin than did PP. With shorter hydrolysis time (24 h), swelling for a longer time led to the increased yield of collagen extracted. Under the same swelling time and hydrolysis time, BSP exhibited the grater extracting ability than did PP. Therefore, both prior swelling time and hydrolysis time affected the collagen extraction from bigeye snapper skin. Electrophoretic study of collagens extracted with different conditions revealed that β, α1 and α2 were the major components. No differences in protein pattern were noticeable among all samples

(Figure 10). Proteins with molecular weight lower than α2-chain was negligible in collagens treated with pepsin. The result indicated that pepsin possibly preferred to cleave the non-helical regions, telopeptides, of collagen structure at low temperature (4°C). Generally, the pepsin activity is enhanced at low pH (Lin and Liu, 2006). At room temperature (26-28°C), pepsin showed high activity and randomly cleaved the swollen collagen molecules of bigeye snapper skin. The integrity of collagen was destroyed under those severe conditions (data not shown).

Table 7 Total hydroxyproline (Hyp) and the yield of collagen extracted from bigeye snapper skin with prior acid swelling, followed by the treatment of bigeye snapper pepsin (BSP) or porcine pepsin (PP) for 24 h and 48 h

Swelling time (h)	Pepsin/hydrolysis time (h)	Total extracted Hyp (mg/g defatted skin)	Yield** (%)
24	-	1.90	7.29±1.20a***
24	BSP/24*	10.66	42.80±0.41d
24	BSP/48	16.19	65.03±0.81g
24	PP/24	10.21	41.02±0.40c
24	PP/48	13.68	54.02±1.06e
48	-	2.31	9.29±0.88b
48	BSP/24	14.79	59.41±1.49f
48	BSP/48	16.22	65.14±0.41g
48	PP/24	11.09	44.52±0.82d
48	PP/48	16.36	65.72±1.22g

Mean±SD from triplicate determinations.

*Numbers denote extraction time (h).

**Yield was calculated based on hydroxyproline content in the collagen in comparison with that of the skin.

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

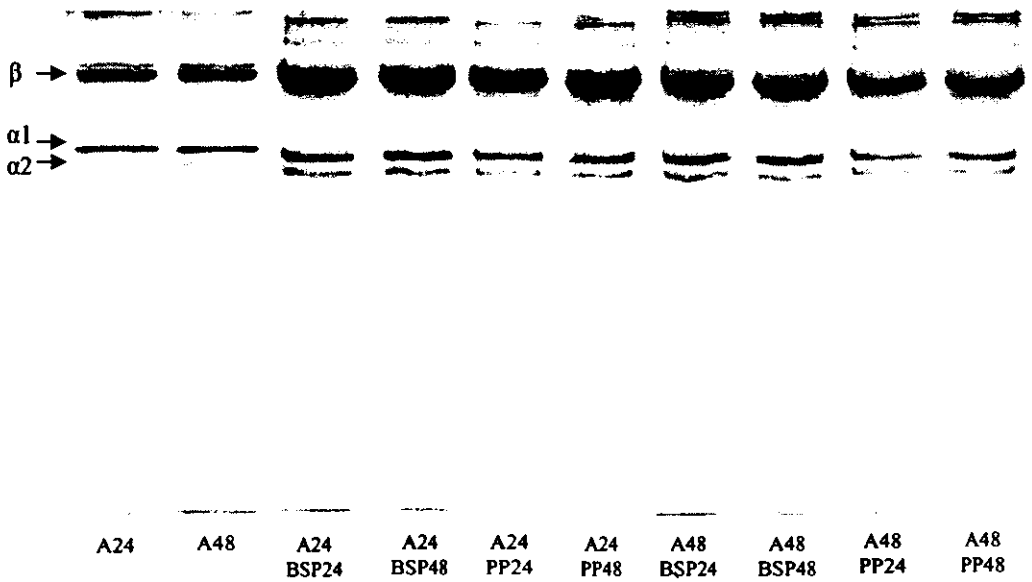

Figure 10 SDS-PAGE patterns of collagen extracted from bigeye snapper skin with prior acid swelling, followed by the treatment of bigeye snapper pepsin (BSP) or porcine pepsin (PP) for 24 h and 48 h.

2.2 Compositions of collagen extracted from bigeye snapper skin with different conditions

The yield, hydroxyproline and collagen contents of different collagens are shown in Table 8. Hydroxyproline and collagen content of bigeye snapper skin were 57.25 and 440.08 mg/g defatted skin, respectively. Approximately 1.5-time increases in hydroxyproline content were obtained in the resultant collagens. The result suggested that the higher content of collagen was found in the extract. The centrifugation after extraction could lead to the removal of non-collagenous substances. As a result, higher hydroxyproline and collagen contents were observed in the extracted collagen, compared with those found in the skin. From the result, the yield of resultant collagens was obviously increased when pepsins were used in extraction process. Greater yield was obtained when BSP was added together with extracting acid medium or after acid swelling process for 24 h. PP showed the lower capacity of extracting collagen from bigeye snapper skin as evidenced by the lower yield. Among all collagen obtained, that extracted using acid without pepsin addition had the lowest yield (5.31%). It was suggested that the swollen skins were easily cleaved with pepsin at telopeptide regions. Therefore, the collagen could be solubilized into the extracting solution in the presence of pepsin to a greater extent, compared with the collagen extracted without pepsin. The different yields (dry weight basis) of pepsin-

solubilized collagen from fish skin were reported in different sources including channel catfish (38.4%) (Liu *et al.*, 2006), ocellate puffer fish (44.7%) (Nagai *et al.*, 2002), black drum (15.8%) and sheephead seabream (29.3%) (Ogawa *et al.*, 2003). Therefore, pepsin had an efficiency in collagen extraction and the increased yield can be obtained.

Table 8 Yield, hydroxyproline and collagen contents of different collagens from bigeye snapper skin

Sample***	Hydroxyproline (mg/g sample)	Collagen* (mg/g sample)	Yield (% dry wt.)
Skin	57.25±0.39a	440.08±1.31a	-
A48	82.74±0.60b	637.09±4.66b	5.31a**
BSP48	83.99±3.04b	646.69±23.43b	18.74c
A24/BSP48	84.08±5.03b	647.39±38.77b	19.79d
A24/PP48	85.14±3.55b	655.57±27.32b	13.03b

Mean±SD from triplicate determinations.

*The conversion factor for calculating the collagen content from hydroxyproline was 7.7 (Kittiphattanabawon *et al.*, 2005).

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

***A48: collagen extracted with acid for 48 h; BSP48: collagen extracted with acid containing BSP (20 kUnits/g defatted skin) for 48 h; A24/BSP48: collagen extracted with acid for 24 h, followed by extracting using BSP (20 kUnits/g defatted skin) for 48 h; A24/PP48: collagen extracted with acid for 24 h, followed by extracting using PP (20 kUnits/g defatted skin) for 48 h.

2.3 SDS-polyacrylamide gel electrophoresis (SDS-PAGE) patterns of collagens from bigeye snapper skin

SDS-PAGE patterns of collagens under reducing and non-reducing conditions are shown in Figure 11. Generally, no differences in protein patterns of collagens under both conditions were observed. The result indicated that all collagens contained no disulfide bond. All collagens comprised β, α1 and α2 chains as the major constituents. The result was accordance with those found in skin collagen of hake and trout (Montero *et al.*, 1990), Japanese sea-bass and

bullhead shark (Nagai and Suzuki, 2000), black drum and sheephead seabream (Ogawa *et al.*, 2004), Pacific whiting (Kim and Park, 2004), young and adult Nile perch (Muyonga *et al.*, 2004a) and channel catfish (Liu *et al.*, 2006). Generally, type I collagen consists of two identical α1 chain and one α2 chain (Rochdi *et al.*, 2000). Based on electrophoretic patterns and subunit composition, it was suggested that collagens obtained from different extraction methods belonged to type I collagen. There were no differences in the relative mobility of α1 and α2 chains between acid-solubilized collagen (A48) and calf skin type I collagen (CSC). However, slight difference in relative mobility between acid-solubilized collagen (A48) collagen and all pepsin-solubilized collagens with different treatments were observed. The molecular weight of α1 and α2 chains in calf skin type I collagen and acid-solubilized collagen was 120 and 112 kDa, respectively. For pepsin-solubilized collagen, including BSP48, A24/BSP48 and A24/PP48, the molecular weight of α1 and α2 chains was estimated to be 118 and 111 kDa, respectively. The results suggested that these pepsin-solubilized collagens might undergo partial cleavage at telopeptide regions by pepsin treatment. As a consequence, a slight decrease in molecular mass of pepsin-solubilized collagens was noticeable, in comparison with that of acid-solubilized collagen. Miller (1972) proposed that the mechanism whereby the proteolytic activity of pepsin alters the solubility properties of cartilage collagen involves, the degradation of non-helical region, thus effectively eliminating a site of intermolecular cross-linking. Drake *et al.* (1966) reported that most of intra- and intermolecular cross-links found in collagen occur through the telopeptide. Some of the telopeptides of calf skin tropocollagen are vulnerable to pepsin action since intramolecular cross-links are broken on pepsin digestion and fragment comprising a small fraction of the molecule (approximately 1%) become dialyzable (Drake *et al.*, 1966). From the result, the higher molecular mass components including γ-chain were found at the higher extent in acid-solubilized collagen (A48), compared with those observed in pepsin-solubilized collagens (BSP48, A24/BSP48, A24PP48). These high molecular mass components might be degraded to smaller components, such as β, α1 or α2, induced by pepsin action. Consequently, the greater band intensity of β, α1 and α2 chains was noticeable in all pepsin-solubilized collagens, compared with acid-solubilized collagen. Similar changes were also found in acid-solubilized calf skin tropocollagen when treated with pepsin. Native calf skin tropocollagen consisted of α: β: γ at the ratio of 32: 65: 3,

whereas the components of pepsin treated tropocollagen were changed to 72: 23: 3 (Drake *et al.*, 1966).

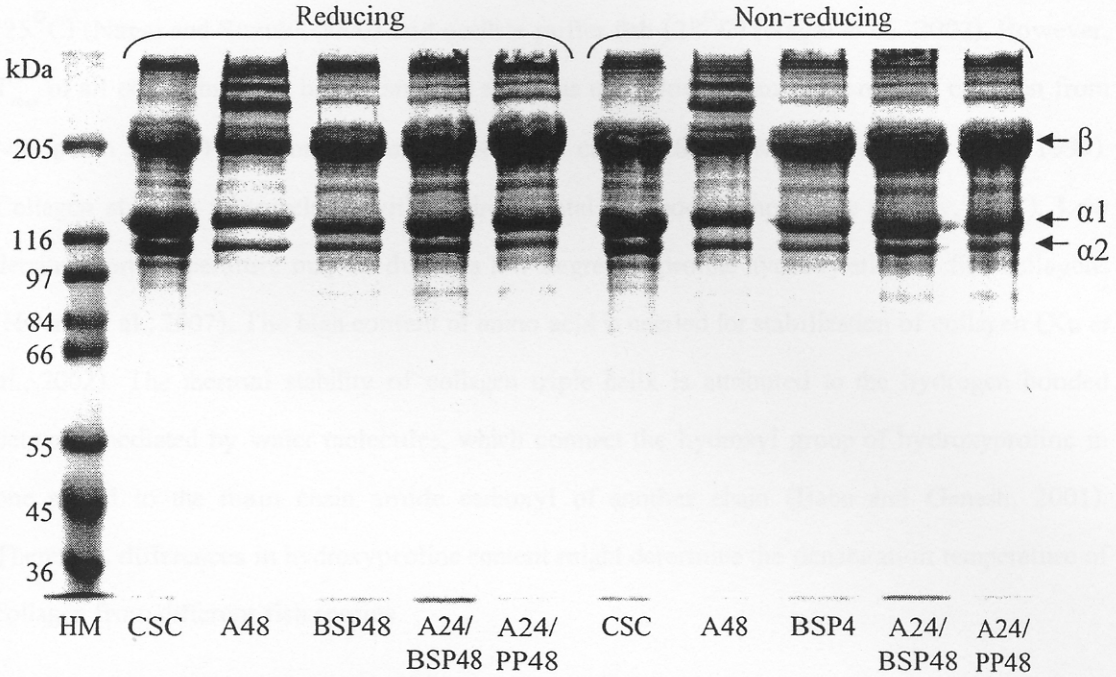

Figure 11 SDS–PAGE patterns of collagens from bigeye snapper skin. A48: collagen extracted with acid for 48 h; BSP48: collagen extracted with acid containing BSP (20 kUnits/g defatted skin) for 48 h; A24/BSP48: collagen extracted with acid for 24 h, followed by extracting using BSP (20 kUnits/g defatted skin) for 48 h; A24/PP48: collagen extracted with acid for 24 h, followed by extracting using PP (20 kUnits/g defatted skin) for 48 h. HM and CSC denote high MW protein markers and collagen type I, respectively.

2.4 Thermal stability of collagens from bigeye snapper skin

Thermal transitions of extracted collagens rehydrated in 0.05 M acetic acid are shown in Table 9. The endothermic peak of acid-solubilized collagen (A48) was observed with T_{max} of 32.5°C, which was slightly higher than that of pepsin-solubilized collagens (BSP48, A24BSP48 and A24PP48) about 1°C. The decrease in denaturation temperature of pepsin-solubilized collagens might be attribute to the molecular weight lowering caused by pepsin digestion, especially at telopeptide region. T_{max} of extracted collagens from bigeye snapper skin

was higher than those previously reported for collagens from several fish species. Different thermal transition temperature of skin collagen have been reported for Pacific whiting (27°C) (Kim and Park, 2004), Japanese sea-bass (26.5°C), chub mackerel (25.6°C), bullhead shark (25°C) (Nagai and Suzuki, 2000) and ocellate puffer fish (28°C) (Nagai *et al.*. 2002). However, T_{max} of all collagens from bigeye snapper skin was much lower than those of skin collagen from Nile perch (36°C) (Muyonga *et al.*, 2004a) and calf (40.8°C) (Komsa-Penkova *et al.*, 1999). Collagen stability is correlated with environmental and body temperature (Rigby, 1968). Low denaturation temperature may be due to a low degree of proline hydroxylation in fish collagens (Hwang et al., 2007). The high content of imino acid is needed for stabilization of collagen (Xu *et al.*, 2002). The thermal stability of collagen triple helix is attributed to the hydrogen bonded network mediated by water molecules, which connect the hydroxyl group of hydroxyproline in one strand to the main chain amide carboxyl of another chain (Babu and Ganesh, 2001). Therefore, differences in hydroxyproline content might determine the denaturation temperature of collagen from different fish species.

Table 9 The maximum transition temperature (T_{max}) and total denaturation enthalpy (ΔH) of different collagens from bigeye snapper skin rehydrated in 0.05M acetic acid

Samples	T_{max} (°C)*	ΔH (J/g)*
A48	32.5	0.106
BSP48	31.5	0.800
A24/BSP48	31.3	0.407
A24/PP48	31.8	1.233

*Values are the mean from duplicate determinations.

A48: collagen extracted with acid for 48 h; BSP48: collagen extracted with acid containing BSP (20 kUnits/g defatted skin) for 48 h; A24/BSP48: collagen extracted with acid for 24 h, followed by extracting using BSP (20 kUnits/g defatted skin) for 48 h; A24/PP48: collagen extracted with acid for 24 h, followed by extracting using PP (20 kUnits/g defatted skin) for 48 h.

2.5 Solubility of skin collagens from bigeye snapper skin

The effect of pH on the solubility of different collagens in 0.5 M acetic acid is depicted in Figure 12. The highest solubility of all collagens was observed at pH 5. Generally, all collagens were highly solubilized in pH range of 2-5 with relative solubility greater than 80%. The sharp decrease in solubility was observed in the neutral pH. However, different collagens had different solubility in the pHs ranging from 6 to 10. Pepsin-solubilized collagen (A24/BSP48) showed the higher solubility than other collagens at pH above 7. This result suggested that A24/BSP48 might possess a lower degree of cross-linking or consisted of the weaker bonds than other collagens. The variation in solubility of collagens might be associated with different isoelectric point (pI), which was altered by pepsin. From the result, it was suggested that pIs of all collagens were more likely in the neutral and alkaline ranges, as evidenced by the lowered solubility in those ranges. At very acidic pH (pH=1). collagen might undergo denaturation to some extent, leading to the impaired solubility. When the pH was closed to pI, the molecular charges of collagen diminished and the decrease in solubility occurred (Vojdani, 1996; Montero *et al.*, 1991). Jongjareonrak *et al.* (2005b) found that acid-solubilzed and pepsin-solubilized collagen from bigeye snapper (*Priacanthus macracanthus*) skin exhibited the lowest solubility at pHs 6 and 7, respectively. Kittiphattanabawon *et al.* (2005) observed the lowest solubility of acid-solubilzed collagen from bigeye snapper (*Priacanthus tayenus*) skin at pH 7.

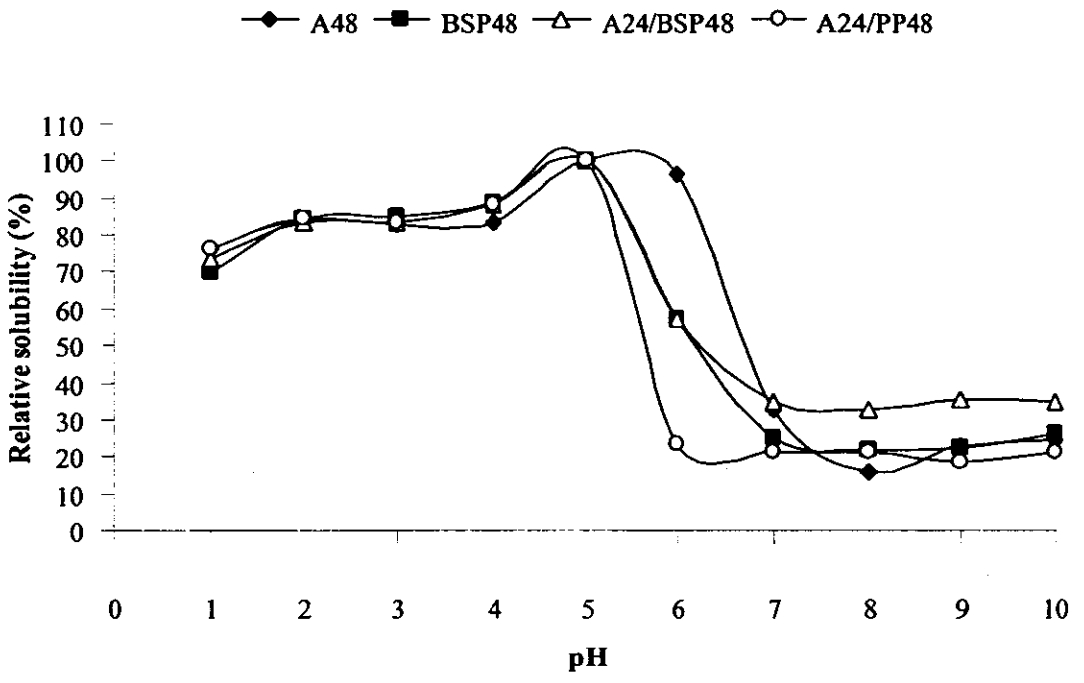

Figure 12 Relative solubility (%) of collagens extracted from bigeye snapper skin with different methods at different pHs.

The effect of NaCl on collagen solubility is shown in Figure 13. The solubility in 0.5 M acetic acid of all collagens was maintained in the presence of NaCl up to 3%. Solubility of all collagens decreased gradually with increasing NaCl concentration. NaCl at higher concentration might result in the decreased protein solubility via 'salting out' effect by increasing hydrophobic interaction and aggregation, and competing with the protein for water. As a result, protein precipitation can be induced (Vojdani, 1996). From the result, pepsin-solubilized collagens (BSP48, A24BSP48 and A24PP48) showed the greater solubility than acid-solubilized collagen (A48), especially at high salt concentration. The descending order of collagen solubility at NaCl above 3% was A24/BSP48, A24/PP48, BSP48 and A48. Greater solubility of pepsin-solubilized collagens might be due to the proteolytic action of pepsin in altering collagen structure and reducing the chain length of resultant collagens. Thus, collagen extracted by different methods might have the different molecular properties, leading to the varied characteristics of resultant collagens. However, all collagens were still soluble more than 50% in the presence of NaCl up to 6%.

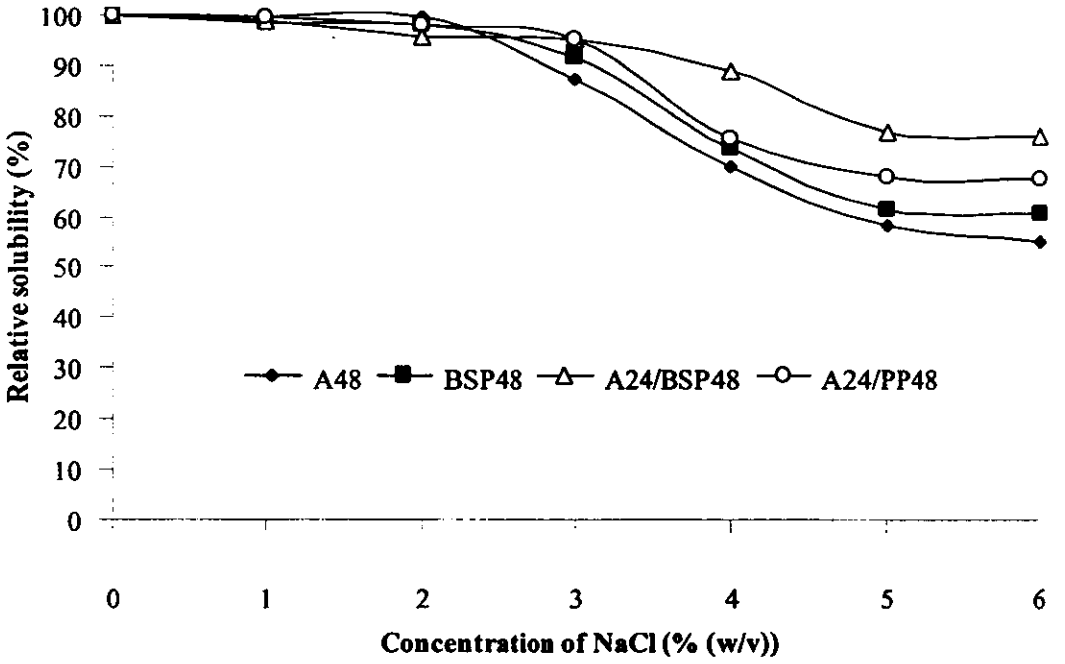

Figure 12 Relative solubility (%) of collagens extracted from bigeye snapper skin with different methods in the presence of NaCl at different concentrations.

3. Effect of pepsin-aided process on extraction and characteristics of gelatin from bigeye snapper skin

3.1 Effect of pepsin levels on extraction and characteristics of gelatin

The gelatin extraction was carried out at 45°C for 12 h after swelling process using 0.2 M acetic acid in the presence of pepsin (BSP) at different levels at 4°C for 48 h. The yield of gelatin expressed as total hydroxyproline content extracted in comparison with that found in bigeye snapper skin is shown in Table 10. The highest yield (40.32%) was obtained when the skin was treated with BSP at 15 kUnits/g alkaline-treated skin. Generally, the yield increased when BSP levels increased. The yield of gelatin extracted with the aid of BSP at 15 kUnits/g alkaline-treated skin was approximately 2-fold higher than that of gelatin extracted without BSP. In general, a mild acid pretreatment of fish skin is usually used prior to gelatin extraction (Montero and Gomez-Guillén, 2000; Gomez-Guillén and Montero, 2001; Gomez-Guillén *et al.*, 2002; Jamilah and Harvinder, 2002). This process disrupts acid labile collagen cross-links with negligible peptide bond hydrolysis and amino acid degradation, resulting in the higher efficacy in gelatin extraction (Slade and Levine, 1987). As an animal matures, there is an increase in amount

of collagen cross-links that are stable to thermal and acid treatment (Galea *et al.*, 2000). These intra- and inter-molecular covalent cross-links containing lysine and hydroxylysine (Hyl) residues and their aldehyde derivatives are mainly located in telopeptide region (Bateman *et al.*, 1996; Gomez-Guillén *et al.*, 2002). Those of cross-links are not solubilized by acid extraction (Jongjareonrak *et al.*, 2005b). Nevertheless, pepsin effectively hydrolyzed a site of intermolecular cross-linking of collagen (Miller, 1972; Drake *et al.*, 1966). Therefore, pepsin might solubilize the collagen in the skin matrix during the acid swelling process. The swollen matrix might be associated with the ease of gelatin extraction. The protein patterns of gelatin solution obtained with the BSP aided process are shown in Figure14. No degradation of β, α1 and α2-chains was obtained in collagen extracted into the acid solution (after 48 h at 4°C). Surprisingly, complete degradation of those components was observed in all gelatin samples. However, degree of hydrolysis was more intense in gelatin from the skin pretreated with BSP, especially with increasing levels. Though the pretreated skins with BSP were neutralized, to inactivated pepsin, those pepsins might activate some endogenous proteinases, which were activated at neutral pH. However, those neutral proteinases did not cause the degradation of collagen under acidic condition and low temperature (4°C). From the result, the proteolytic activity was enhanced at 7.5 and 45°C, the condition used for gelatin extraction. Therefore, these proteinases were more likely to be the neutral or alkaline proteinases. The same result has been reported in gelatin from bigeye snapper (*Priacathus macracanthus*) skin extracted without proteinase inhibitors (Intarasirisawat *et al.*, 2006). Endogenous skin proteases have been classified to be serine protease, which was inhibited by soybean trypsin inhibitor (SBTI) (Intarasirisawat *et al.*, 2006). Effect of extraction times at 45°C on protein patterns of gelatin extracted from pretreated skin using 15 kUnits BSP/g alkaline-treated skin is shown in Figure 15. The major components (β, α1 and α2) of gelatin were dramatically degraded to lower MW components after 2 h of extraction. Complete degradation of gelatin was observed at 4 h of extraction. Due to the higher efficacy of BSP at 15 kUnits/g skin in cleaving the telopeptide of collagen prior to gelatin extraction, it was most likely to extract the gelatin more easily. Therefore, BSP at a level of 15 kUnits/g alkaline-treated skin was chosen for further study. To retain the major components in gelatin, the inactivation of the skin endogenous proteases active at neutral or alkaline pHs at 45°C was required.

Table 10 Hydorxyproline (Hyp) yield of gelatin extracted from bigeye snapper skin treated with bigeye snapper pepsin (BSP) at different levels

BSP levels (kUnits/g alkaline-treated skin)	Total extracted Hyp* (mg/g alkaline-treated skin)	Yield** (%)
0	4.40±0.07a	22.16±0.35a
5	6.71±0.17b	33.85±0.85b
10	7.47±0.13c	37.66±0.66c
15	8.00±0.09d	40.32±0.44d

Mean±SD from triplicate determinations.

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

**Yield was calculated based on hydroxyproline content in the gelatin in comparison with that of the skin.

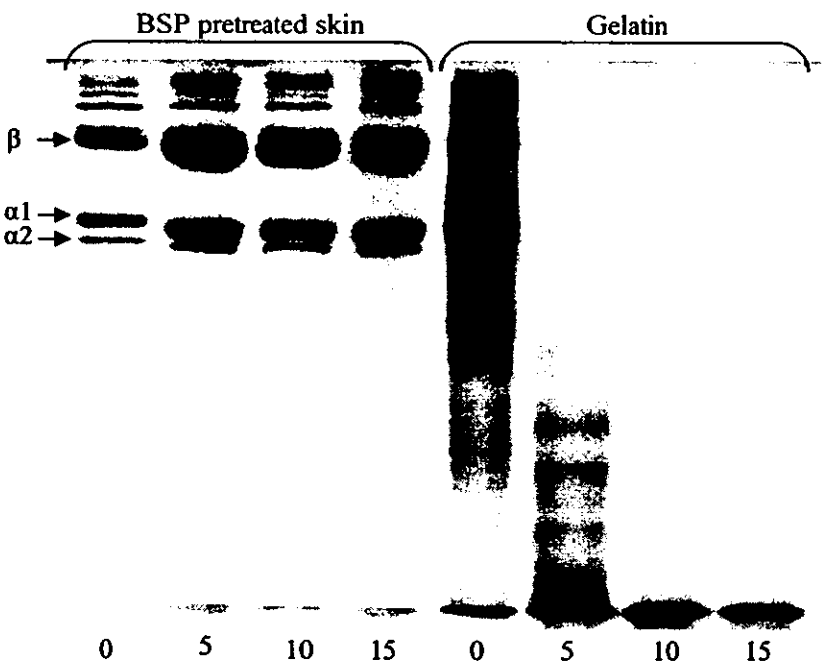

Figure 14 SDS-PAGE patterns of BSP pretreated skin and gelatin extracted from bigeye snapper treated with BSP at different levels. Skin was pretreated in acid solution at 4°C for 48 h in the presence of BSP at different levels. Gelatin was extracted after pretreatment at 45°C for 12 h. Number denote activity levels (kUnits/g alkaline-treated skin).

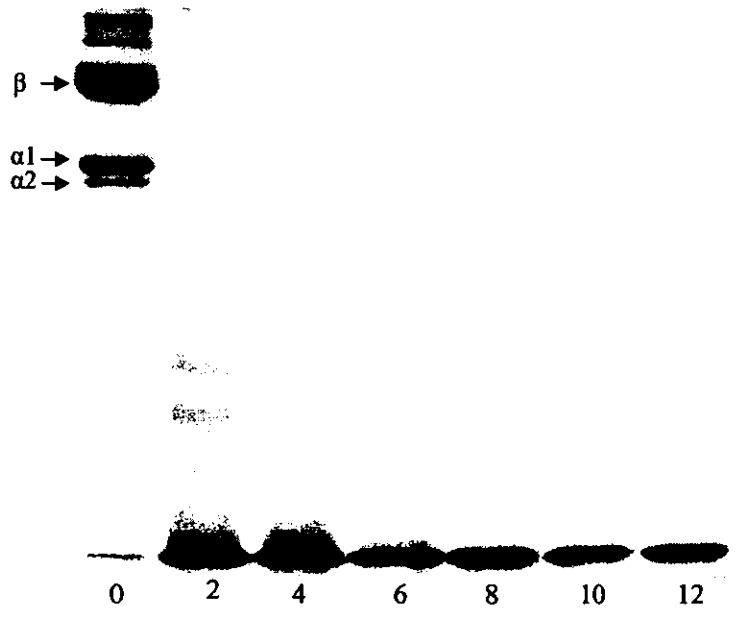

Figure 15 SDS-PAGE patterns of gelatin extracted from bigeye snapper skin, treated with BSP at 15 kUnits/g alkaline-treated skin, at 45°C for different times. Numbers denote time of gelatin extraction (h).

3.2 Inactivation of endogenous proteases in BSP pretreated bigeye snapper skin

Uses of heat treatments for inactivation of endogenous proteinases in BSP treated skin were studied. The SDS-PAGE patterns of gelatin extracted from BSP treated skin, subjected to heating at different temperatures, are shown in Figure 16. Although the major components (β, α1 and α2) of gelatin molecules were retained to some extent after heat treatments, the degradation of the lower component β, α1 and α2-chains still occurred as evidenced by the formation of low MW components. From the result, the higher temperature used resulted in the greater extent of β or α2-chains retained in the gelatin extracted. This was probably due to the partial inactivation of those proteinases at high temperature. The results revealed that skin endogenous proteases were quite stable at high temperature. These enzymes were considered to be heat-stable proteinases, which still remained their activity after heating up to 90°C at pH 7.5. Heat-stable alkaline proteinases required some kind of activation for the *in vitro* assay such as heating, gamma irradiation or treatment with several reagents (urea, SDS, some fatty acids, poly-L-lys or alkylating agents) in order to expose their active sites (Makinodan *et al.*, 1987; Wilk and Orlowski, 1983).

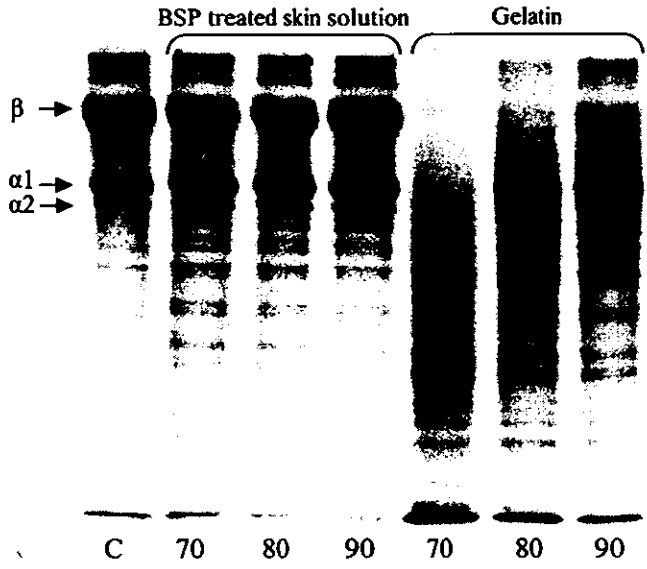

Figure 16 SDS-PAGE patterns of BSP pretreated skin heated at different temperatures for 5 min and gelatins extracted from BSP pretreated skin heated at different temperatures. Gelatin was extracted at 45°C for 12 h. C denote swollen skin treated with bigeye snapper pepsin before heat-treatment.

To prevent the degradation of major components of gelatin molecule, pepstatin A and soybean trypsin inhibitor at different concentrations were incorporated during extraction at 45°C for 12 h. To monitor the degradation, protein patterns of gelatin extracted in the absence and the presence of different proteinase inhibitors were determined (Figure 17). From the result, pepstatin A had no inhibitory effect on the skin endogenous proteases as shown by the complete disappearance of major components. Similar result was observed in gelatin extracted without inhibitor (lane C). The result also reconfirmed that no pepsin involved in the degradation of gelatin molecule. The skin endogenous proteases activity was strongly inhibited by soybean trypsin inhibitory (SBTI), which could prevent the degradation of the major components involving β, α1 and α2 chains of gelatin. The result indicated that endogenous proteinases in bigeye snapper (*P. tayenus*) skin were most likely serine proteinase. The result was in accordance with Intarasirisawat *et al.* (2006) who reported that serine proteinase was the major enzyme causing the autolysis of bigeye snapper skin. Moreover, heat-stable alkaline proteinase from *P. macracanthus* muscle was also strongly inhibited by soybean trypsin inhibitor (Benjakul *et al.*, 2003b).

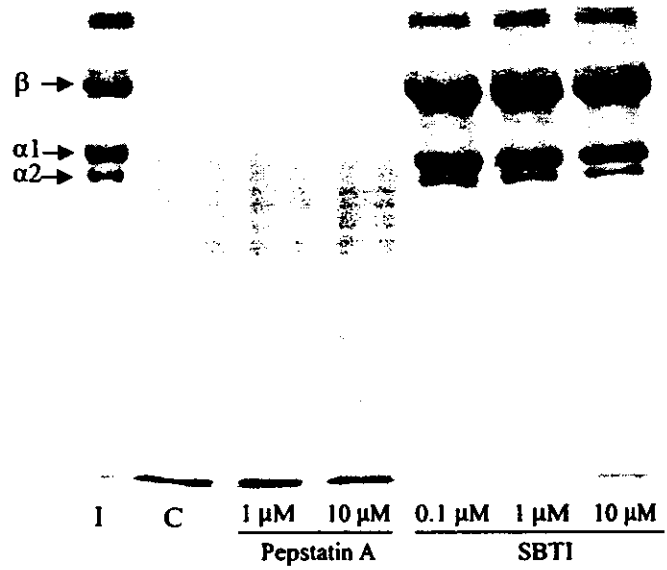

Figure 17 SDS-PAGE patterns of gelatin extracted from bigeye snapper skin in the absence and the presence of proteinase inhibitors at different concentrations. I and C denote collagen type I and gelatin extracted without proteinase inhibitor.

3.3 Proximate compositions of bigeye snapper skin and gelatins

Proximate compositions of bigeye snapper skin and gelatins are shown in Table 11. Bigeye snapper skin constituted water (65.67%) as a major component, followed by protein (23.14%), ash (10.92 %) and trace amount of fat (0.38%). The compositions of skin generally vary with fish species. Cheow *et al.* (2007) reported that moisture, protein, fat and ash contents of sin croaker skin were 62.33%, 24.8%, 7.99% and 5.43%, respectively, and were 60.93%, 24.1%, 9.63% and 5.9%, respectively, for shortfin scad skin. Gelatins extracted from different methods had different compositions. From the result, gelatin extracted from bigeye snapper skin by typical process (GT) had the similar compositions to gelatin from bovine bone (GB). Both gelatins contained high protein content (83.78% and 82.49%) with low moisture content (10.01% and 10.94%). Ash content of GT (5.77%) was higher than that of gelatin from other species including brownstripe red snapper skin gelatin (1.9%) (Jongjareonrak *et al.*, 2006), sin croaker skin gelatin (1.49%) and shortfin scad skin gelatin (1.15%) (Cheow *et al.*, 2007). The result suggested that high ash content might be from the scales found in the skin used for gelatin extraction. The appropriate demineralization of bigeye snapper skin should be carried out prior to gelatin extraction

to reduce ash content. Generally, no differences in compositions and yield were observed between gelatins extracted from bigeye snapper skin treated with BSP and porcine pepsin (PP). However, it was noted that low protein content was observed in gelatin extracted from bigeye snapper skin treated with BSP (GA) (43.37%) and PP (GP) (43.21%). Coincidentally, these gelatins contained high ash content (~47%).

Table 11 Proximate compositions of bigeye snapper skin and different gelatins

Sample	Compositions* (% wet wt.)				Hydroxyproline content (mg/g sample)	Yield*** (%)
	Moisture	Protein	Fat	Ash		
Skin**	65.57±0.47^{d}	23.14±0.58^{a}	0.38±0.05^{a}	10.92±0.72^{b}	19.50±0.42^{a}	-
GT	10.01±0.24^{b}	83.78±0.33^{d}	0.44±0.04^{ab}	5.77±0.55^{a}	90.80±0.53^{c}	25.67±0.15^{a}
GA	8.83±0.55^{a}	43.37±0.35^{b}	0.43±0.03^{ab}	47.37±0.34^{c}	30.55±0.78^{b}	36.99±0.94^{b}
GP	9.03±0.47^{a}	43.21±0.66^{b}	0.46±0.07^{ab}	47.30±0.36^{c}	29.63±0.26^{b}	37.33±0.33^{b}
GB	10.94±0.15^{c}	82.49±0.53^{c}	0.52±0.06^{b}	6.05±0.60^{a}	115.71±0.71^{d}	-

GT: gelatins extracted using typical method; GA: gelatins extracted from bigeye snapper skin treated with BSP; GP: gelatins extracted from bigeye snapper skin treated with porcine pepsin (PP); GB: gelatin from bovine bone.

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

*Mean±SD from triplicate determinations.

**Skin with non-collagenous protein removal.

***Yield was calculated based on hydroxyproline content in the gelatin in comparison with that of the skin.

High ash content in these gelatins might result from the salt formed during the neutralization of acidic solution using NaOH to inactivate pepsin before gelatin extraction. Thus, the appropriate desalting of those gelatins before drying should be done to reduce the ash content. Simon *et al.* (2002) reported that the salinity of the fish gelatin was reduced by 33% with diafiltration technique (DF) at an average desalting speed of 185 g/h.m^{2} with a few loss of protein in the permeate side. The hydroxyproline content of bigeye snapper skin gelatins extracted using

typical method (GT) was lower than that of GB. The imino acid content of gelatins has a strong influence on their functional properties (Gilsenan and Ross-Murphy, 2000). Based on hydroxyproline extracted, a greater yield was obtained when BSP or PP was used in combination with acid swelling process. The result suggested that the cross-linked molecules at telopeptide regions of collagen were cleaved by pepsin, both BSP and PP, resulting in the greater extractability of gelatin at high temperature (45°C).

3.4 Protein patterns of different gelatins

SDS-PAGE patterns of different gelatins under reducing and non-reducing condition are shown in Figure 18. All gelatins were characterized to be type I without disulfide bond. For the same gelatin, no differences in protein patterns were observed between reducing and non-reducing conditions. In general, protein patterns of different gelatins were slightly different. Commercial gelatin from bovine bone (GB) comprised α-chain as the major components with the molecular weight ranging from 118 to 123 kDa. Nevertheless, the low molecular weight molecules were also observed, suggesting the cleavage of peptides during gelatin preparations. In gelatin manufacture, the conversion of collagen to gelatin yields the molecules of varying mass, due to the cleavage of inter-chain chemical cross-links and some unfavorable breakage of inter-chain peptide linkage (Zhou *et al.*, 2006). Gelatin from bigeye snapper skin extracted by typical process (GT) consisted of α1 chain as the major constituent with molecular weight of 119 kDa. Low molecular weight components were also observed, particularly with the molecular weight of 83 kDa. The result suggested that a gelatin molecule was degraded during extraction with hot water (45°C). Skin endogenous proteases, especially heat-activated protease or collagenolytic enzymes, might involve in the degradation. Generally, gelatin extracted from bigeye snapper skin treated with BSP (GA) and PP (GP) were composed of β and α components. However, GP contained 2 major bands with molecular weight less than α2 chain (110 and 102 kDa). The result suggested that the difference in molecular weight distribution of resulting gelatin was possibly associated with the different specificity of both pepsins in hydrolyzing gelatin molecules. BSP had the greater specificity to cleave inter-chain cross-links of native collagen than did PP since the major components were more retained when BSP was used. Muyonga *et al.* (2004b) reported that the functional properties of gelatin appeared to be related to difference in molecular weight distribution of gelatin.

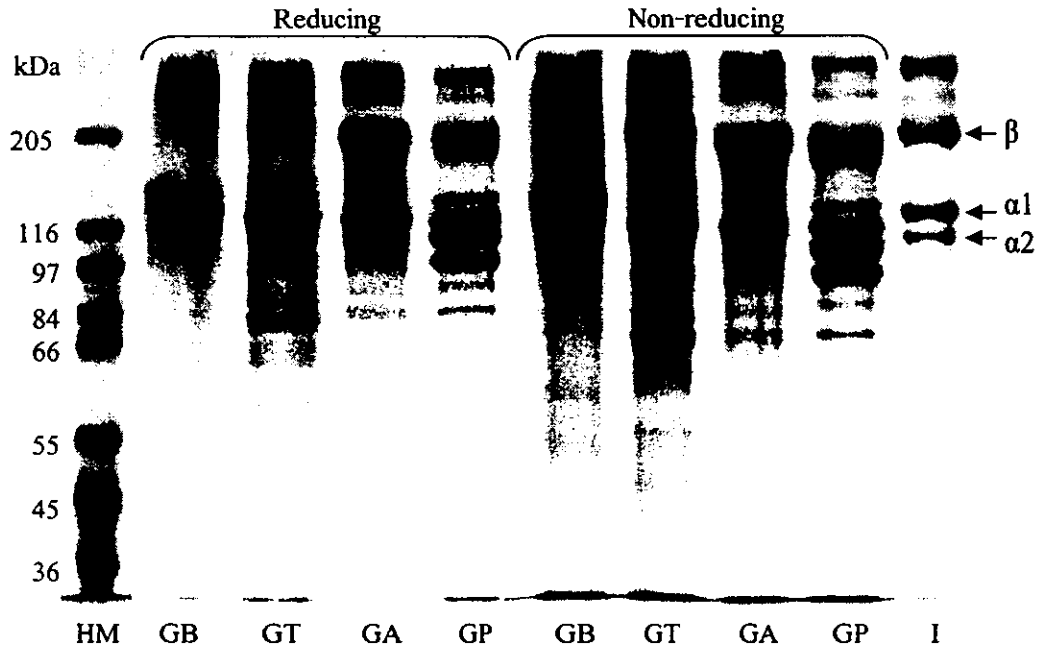

Figure 18 SDS–PAGE patterns of gelatin from bigeye snapper skin extracted by typical process (GT); gelatin extracted from skin treated with bigeye snapper pepsin (GA) and porcine pepsin (GP) at levels of 15 kUnits/g skin and gelatin from bovine bone (GB) under reducing and non-reducing conditions. HM and I denote high MW protein markers and collagen type I, respectively.

3.5 Gel characteristics of different gelatins

The color (L*, a* and b*-values) and bloom strength of the gels from different gelatins are shown in Table 12. Generally, the color of gelatin gels from bigeye snapper skin varied, depending on the process used. Pepsin-aided process resulted in the lower L-values, possibly due to the darker color of enzyme solution added during extraction process. Higher L*-value of gelatin gel from commercial food grade gelatin (GB) was observed. Gelatin manufacture generally has a good process to clarify the impurities from gelatin solution such as chemical clarification and filtration process (Ward and Courts, 1977). Nevertheless, the higher redness (a*-value) and yellowness (b*-value) were found in GB when compared with bigeye snapper skin gelatins. The result indicated that the color of gelatin gel was dependent on the sources of gelatin

as well as process used. However, the color did not affect other functional properties (Ockerman and Hansen, 1988).

Table 12 Color and bloom strength of gel and turbidity of gelatin solution (6.67% protein) from different gelatins*

Samples	Color			Turbidity	Bloom
	L*	a*	b*	(A_{360})	(g)
GT	57.79±0.10c**	1.69±0.14b	11.14±0.08c	2.238±0.037c	56.0±1.3a
GA	53.73±0.09a	1.97±0.05b	10.09±0.03b	1.699±0.015b	135.3±2.7b
GP	55.46±0.19b	1.08±0.02a	6.88±0.09a	1.707±0.013b	138.6±1.8b
GB	67.91±0.56d	3.05±0.29c	23.08±0.12d	0.561±0.012a	250.0±0.6c

*Mean±SD from triplicate determinations.

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

As expected, GB showed the higher bloom strength (250 g) than did bigeye snapper skin gelatins. Hydroxyproline content of GA and GP were around 7% when compared with its protein content, while that of GB was 14% (Table 11). The gelling properties of gelatin were also influenced by the source of raw materials, which vary in proline and hydroxyproline contents (Jongjaronrak *et al.*, 2006). The main structural difference between fish and mammalian gelatins is the imino acid contents (proline and hydroxyproline), where the mammalian gelatins have the higher amount (Gudmundsson, 2002). Similar gel strengths of GA and GB were observed ($P>0.05$) with the bloom of 135.3 and 138.6 g, respectively. Bloom strength of these gelatin gels was higher than other fish skin gelatins previously reported including Alaska pollock gelatin (98 g) (Zhou *et al.*, 2006) and sin croaker gelatin (124.94 g) (Cheow *et al.*, 2007). The gel strength and melting point of the gels prepared from fish gelatin decreased with increasing NaCl concentration (Choi and Regenstein, 2000). Due to high salt concentration in GA and GB as shown by high ash content, it might lower gel forming ability by increasing electrostatic repulsions between gelatin molecules. The greater gel strength may be obtained if desalination process is used. Haug *et al.* (2004) reported that the storage modules (G') of fish gelatin increased

at low ionic strengths, while decreasing at medium to high salt concentrations. They suggested that electrostatic interactions are important in the stabilization of the gelatin gel network. Choi and Regenstein (2000) observed that NaCl decreased the gel strength of gelatin from different sources via breaking both hydrophobic and hydrogen bonds. As a consequence, the formation of junction zones in gel network could be prevented. Among all samples, the lowest gel strength was observed for GT (56 g). This was probably associated with the lower gel forming ability of this gelatin caused by the shorter chain length of gelatin molecules. As a result, the weaker gel network was presumably formed.

Protein patterns of all gelatins are shown in Figure 19. Similar protein patterns were observed in GA, GB and GP before and after gel setting. Conversely, GT showed substantial degradation of the compounds found in gelatin after dissolving the sample at 60°C for 20 min before gel maturation. These degraded gelatin molecules possibly formed the small aggregate or cluster, leading to the turbidity of GT solution. When protein was treated for a long time at high temperature, aggregation is activated and turbidity increased (Johnson and Zabik, 1981). The high turbidity of GA and GP might be due to the formation of aggregates of high-molecular-weight compounds in the presence of high salt content. The lowest turbidity was found in GB, which was purified for industrial use to improve the clarity. Muyonga *et al.* (2004b) reported that turbidity values are largely dependent on the efficiency of clarification (filtration) process.

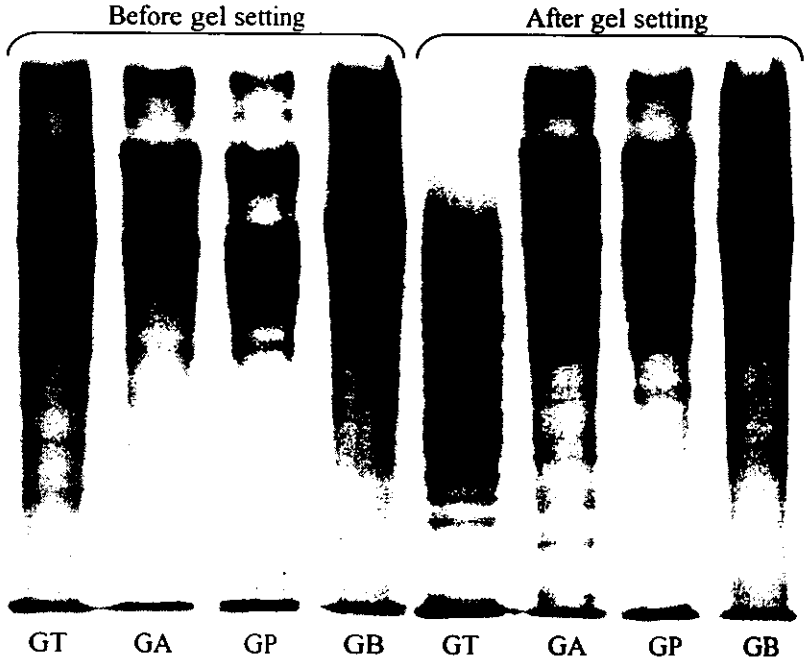

Figure 19 SDS–PAGE patterns of gelatin from bigeye snapper skin extracted by typical process (GT); gelatin extracted from skin treated with bigeye snapper pepsin (GA) and porcine pepsin (GP) and gelatin from bovine bone (GB) before and after gel setting.

3.6 Solubility of gelatins

The effect of pH on solubility of gelatins is depicted in Figure 20. The minimal solubility of GT was found at pH 7. Normally, gelatin type A and acid-processed gelatin have isoelectric points varying from 6.5 to 9.0 (Foegeding *et al.*, 1996; Johnston-Banks, 1990). During acid hydrolysis of gelatin, some glutamine and asparagine will be converted to the acidic forms, i.e. glutamic acid and aspartic acid, respectively. (Jamilar and Harvinder, 2002). Eastoe and Leach (1977) proposed that deamination of asparagine and glutamine occurs during prolonged exposure of collagenous material to acid or alkaline, leading to the decrease in pI values. The maximal solubility of GA and GP was observed at pH 3 and 8, respectively. The differences in solubility of GA and GP were possibly caused by the alteration of gelatin structure induced by pepsin used. Generally, solubility of GB was greater than that of bigeye snapper skin gelatin extracted with the typical method at pHs range of 1-10. The difference in solubility of different gelatins might result from the differences in molecular weight and the content of polar and non-

polar groups in amino acids (Zayas, 1997). However, all gelatins showed high solubility at pHs range of 1-10 with relative solubility more than 80%.

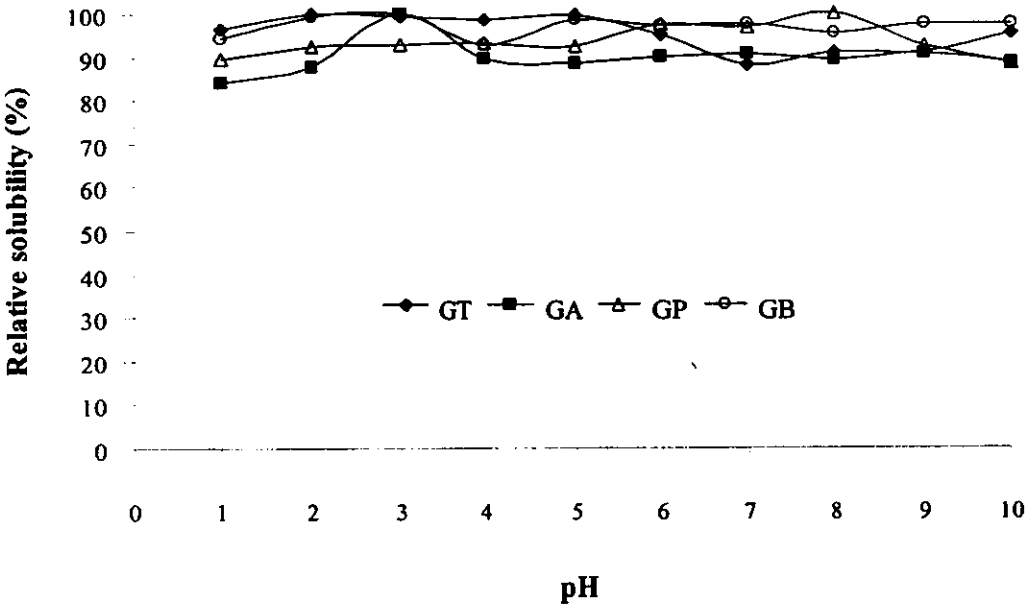

Figure 20 Relative solubility of different gelatins from bigeye snapper skin. GT, GA and GP denote gelatin extracted by typical process, gelatin extracted from skin treated with bigeye snapper pepsin and and porcine pepsin, respectively. GB denote gelatin from bovine bone.

3.7 Emulsifying properties

Emulsion activities index (EAI) and emulsion stability index (ESI) of gelatin from bigeye snapper skin (GT, GA and GP) and food grade gelatin from bovine bone (GB) at protein concentration of 0.1% (w/v) are shown in Table 13. Generally, both EAI and ESI of GA and GP were similar. GB also exhibited similar EAI to those gelatins. However, GB showed higher emulsion stability (lower ESI value). The differences in emulsifying properties of gelatin from different sources were the result of the varying intrinsic properties, compositions and conformation of different gelatins. GT showed the highest EAI when compared with other gelatins. The result suggested that lower MW gelatin molecules of GT with high solubility could migrate to the surface of fat droplet more potentially than other gelatins. Lin and Chen (2006) proposed that the emulsification process includes two steps: 1) deformation and disruption of

droplets which increase the specific surface area of emulsion and 2) the stabilization of this newly formed interface by surfactant. As a result, those GT molecules could arrange at the interface and stabilize the oil droplet to a higher extent. However, the lowest ESI was observed in GT. The lower-molecular-weight molecules possibly exhibited the lower steric forces to stabilize oil droplet. Therefore, emulsifying properties of gelatin were governed by molecular properties, particularly the size of peptides.

Table 13 Emulsifying properties and foaming properties of gelatin from bigeye snapper skin and gelatin from bovine bone

Sample	Emulsifying properties*		Foaming properties**	
	EAI	ESI	Foam ability	Foam stability
GT	51.08±3.49b***	96.19±0.17c	1.21±0.18a	0.76±0.07b
GA	43.96±4.61a	84.17±3.64b	2.68±0.25b	0.58±0.11a
GP	40.02±3.56a	83.89±1.33b	1.74±0.20a	0.51±0.02a
GB	39.92±1.92a	76.92±5.43a	4.10±0.34c	0.55±0.01a

Mean±SD from triplicate determinations.

*Gelatin solution with 0.1% protein content.

**Gelatin solution with 1.0% protein content.

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

3.8 Foaming properties

Form formation ability and foam stability of different gelatins at concentration of 1% protein are shown in Table 13. The greatest foam formation ability was observed in GB with the ratio of foam volume/liquid volume of 4.1. Foam formation ability of gelatin from bigeye snapper skin prepared from different methods varied from 1.2 to 2.6. Foam stability of GB was similar to GA and GP with the ratio of foam volume after 30 min/initial foam volume about 0.5. Foam stability of GT was slightly higher than that of other gelatins. The result suggested that the decrease in foam formation and stability might be due to the aggregation of proteins, which interfered with the interaction between the protein and water needed for foam formation

(Kinsella, 1997; Cho *et al.*, 2004). Zayas (1997) reported that the properties of proteins, which enable them to form stable films in foam, are affected by the molecular configuration of proteins, their intermolecular bonds, and the content and disposition of hydrophobic residues.

3.9 Microstructure of gelatin gels

The microstructure of gelatin gels from bigeye snapper skin (GT, GA and GP) and bovine bone (GB) was visualized by SEM as shown in Figure 21. In general, GB gel showed the finer and denser strands, compared with those of bigeye snapper gelatin gels. This was possibly due to the different characteristics of gelation between species in terms of aggregation and alignment of protein molecules (Jonjareonrak *et al.*, 2006). GT gel showed the looser structure with the larger void than other gelatin gels, possibly caused by proteolysis of gelatin molecules during gel preparation (Figure 19). Gelatins with the shorter chain tended to form the junction zone to a lower extent. As a consequence, the interconnections of gel network were lowered. The differences in microstructure of GA and GP were observed even though it had the similar bloom strength. GA gel contained smaller voids than did GP gel. The result suggested that different molecular distribution and properties between GA and GP might contribute to the different gel network formation. Thus, different alignment or aggregation of gelatin molecules might govern the bloom strength of gelatin gel differently. Zayas (1997) reported that two gelatins with the same average molecular weight from similar sources and identical maturation may have different gel strength. The two main reasons of that are heterogeneity, with regard to size and shape of the molecules and also the difference in the amount and distribution of amino acids within the poly peptide chain. Furthermore, the dislocation and concentration of the proline and hydroxyproline in the collagen chains influences the rate of gel formation and the textural properties, especially the strength of the gel (Zayas, 1997).

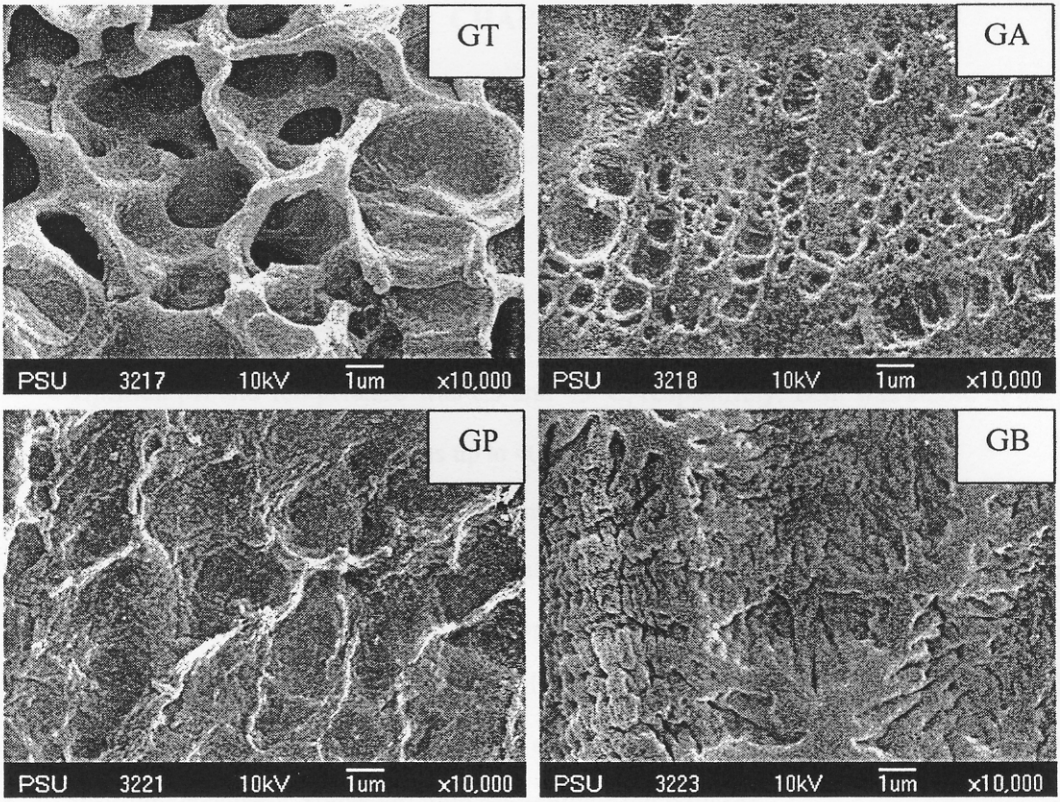

Figure 21 Microstructure of gelatin gels (magnification: 10,000 X). GT: gelatin gel of bigeye snapper skin extracted by typical process. GA and GP: gelatin gels of bigeye snapper skin extracted by pepsin-aided process using with bigeye snapper pepsin and porcine pepsin, respectively. GB: gelatin gel of bovine bone.