CHAPTER 2

ISOLATION AND CHARACTERIZATION OF COLLAGEN FROM BIGEYE SNAPPER (*PRIACANTHUS MACRACANTHUS*) SKIN

2.1 Abstract

Acid-solubilized collagen (ASC) and pepsin-solubilized collagen (PSC) were isolated from the skin of bigeye snapper (*Priacanthus macracanthus*) with the yields of 6.4% and 1.1% on the basis of wet weight, respectively. Both ASC and PSC were characterized to be type I with no disulfide bond. Peptide maps of ASC and PSC digested by V8 protease and lysyl endopeptidase showed some differences in peptide patterns and were totally different from those of calf skin collagen. The maximum solubility was observed at pH 4 and 5 for ASC and PSC, respectively. The sharp decrease in solubility of both collagens in acetic acid was found with NaCl concentration above 3%. Thermal transitions of ASC and PSC in deionized water were observed with the T_{max} of 30.37 and 30.87°C, respectively, and were lowered in the presence of 0.05 M acetic acid. Therefore, ASC was a major fraction in bigeye snapper skin and it exhibited some different characteristics, compared to PSC.

2.2 Introduction

Collagen is a major fraction of connective tissues such as tendon, skin, bone, the vascular system of animals, and the connective tissue sheaths surrounding muscle (Foegeding *et al.*, 1996). Furthermore, collagen has been found in fish skin, bone, and scale (Kimura, 1992; Nagai and Suzuki, 2000c; Nomura *et al.*, 1996; Ikoma *et al.*, 2003) as well as in shellfish tissue (Mizuta *et al.*, 1994; 1996; Nagai *et al.*, 2001; Nagai *et al.*, 2002). Collagen and gelatin have been widely used in food industries as ingredients to improve the elasticity, consistency and stability of foods. It can be used for encapsulation and edible film formation, making it of interest to the pharmaceutical, biomaterial-based packaging, and photographic industries (Stainsby, 1987; Tabata and Ikada, 1998; Sobral *et al.*, 2001; Arvanitoyannis *et al.*, 1997; 1998b). Collagen and gelatin of land animal origin such as bovine and porcine skins and bones have been mainly

used (Yoshimura *et al.*, 2000). However, the outbreak of mad cow disease has resulted in anxiety among users of cattle gelatin. Additionally, the collagen obtained from pig skin or bone cannot be used as a component of some foods due to esthetic and religious objections (Sadowska *et al.*, 2003). Therefore, alternative sources, such as fish processing waste including skin, bone or scale, have been paid increasing attention for collagen and gelatin extraction.

Surimi is one of important income generators for Thailand, which are exported to several countries. Most of fish species used for the production are threadfin bream (*Nemipterus* spp.), bigeye snapper (*Priacanthus* spp.), croaker (*Pennahia* and *Johnius* spp.) and lizardfish (*Saurida* spp.) (Benjakul *et al.*, 2003). During processing, a large amount of wastes is generated. Fish solid wastes constitute 50-70% of the original raw material, depending on the method of meat extraction from the carcass (Morrissey *et al.*, 2000). This waste is an excellent raw material for the preparation of high-protein foods, besides helping to eliminate harmful environmental aspects (Shahidi, 1994). About 30% of such wastes consist of skin and bone with high collagen content (Gomez-Guillen *et al.*, 2002). Nagai and Suzuki (2000a) reported that the collagen content in fish waste skin of Japanese sea-bass, chub mackerel, and bullhead shark were 51.4%, 49.8%, and 50.1%, respectively, on the basis of lyophilized dry weight. Collagen contents vary, depending on fish species (Nagai *et al.*, 2002; Ciarlo *et al.*, 1997; Montero *et al.*, 1991). Type I collagen has been found as the major collagen in fish waste material skin, bone, and fins of various fish species (Kimura, 1983; 1992; Nagai and Suzuki, 2000a; Nagai *et al.*, 2002; Sato *et al.*, 1989). Recently, collagen subunits from shark skin and sea cucumber (Nomura *et al.*, 1995; Saito *et al.*, 2002) have been characterized. Nevertheless, collagen from different species and habitats might be different in terms of molecular compositions and properties (Foegeding *et al.*, 1996). So far, a little information regarding the characteristics of marine fish skin collagen, especially from commercially important species including those used for surimi production, has been reported. Therefore, the objective of this investigation was to isolate and characterize collagen from the skin of bigeye snapper (*Priacanthus macracanthus*) which is one of the abundant fish species used for surimi production in Thailand.

2.3 Materials and Methods

Chemicals

β-mercaptoethanol (β-ME) and protein marker were purchased from Sigma Chemical Co. (St. Louis, Mo, USA). Sodium dodecyl sulfate (SDS), acetic acid, and Tris(hydroxymethyl)aminomethane were obtained from Merck (Darmstadt, Germany).

Fish skin preparation

Bigeye snapper (*Priacanthus macracanthus*) with an average total length of 22-25 cm were caught from Songkhla coast along the Gulf of Thailand, stored in ice and off loaded after 24-36 h of capture. Upon arrival to the dock in Songkhla, fish were stored in ice with a fish per ice ratio of 1:2 (w/w) and transported to the Department of Food Technology, Prince of Songkla University, Hat Yai. Fish were washed using a tap water. Skins were then removed, descaled, and cut into small pieces (0.5x0.5 cm). Skins were kept on ice prior to collagen extraction.

Skin collagen preparation

The collagen was extracted according to the method of Nagai and Suzuki (2000a) with a slight modification. All processes were carried out at 4°C. Skin was soaked in 0.1 M NaOH with a sample per solution ratio of 1:30 (w/v) for 24 h with a gentle stirring. The solution was changed every 8 h to remove noncollagenous proteins and pigments. Alkali treated skins were then washed with distilled water until neutral or faintly basic pHs of wash water were obtained. Fat was removed in 10% (v/v) butyl alcohol with a sample per solution ratio of 1:30 (w/v) for 24 h with a gentle stirring and a change of solution every 8 h. Defatted skins were washed with distilled water thoroughly. The matter was soaked in 0.5 M acetic acid with a sample per solution ratio of 1:30 (w/v) for 24 h with a gentle stirring. The mixture was then centrifuged at 20,000 x *g* for 1 h. The supernatants were collected and kept at 4°C. The precipitate was re-extracted in 0.5 M acetic acid with a sample per solution ratio of 1:30 (w/v) for 16 h with a gentle stirring, followed by centrifugation at 20,000 x *g* for 1 h. The supernatants obtained were

combined with the first extract and the solution was precipitated by the addition of NaCl to a final concentration of 2.6 M in 0.05 M Tris-HCl (pH 7.5). The resultant precipitate was collected by centrifugation at 20,000 x *g* for 1 h and then dissolved in 10 volumes of 0.5 M acetic acid. The solution obtained was dialyzed with 10 volumes of 0.1 M acetic acid in a dialysis membrane with molecular weight cut off of 30 kDa for 12 h at 4°C with change of solution every 4 h. Subsequently, the solution was dialyzed with 10 volumes of distilled water with change of water until neutral pH was obtained. Dialysate was freeze-dried and referred to as acid-solubilized collagen (ASC). Undissolved residue obtained after acid extraction was thoroughly rinsed with distilled water, suspended in 2 volumes of 0.5 M acetic acid and subjected to the limited hydrolysis with 10% (w/v) pepsin (EC 3.4.23.1; powderized; 750 U/mg dry matter, Sigma Chemical Co. (St. Louis, Mo, USA)) for 48 h at 4°C with a gentle stirring. The viscous solution was centrifuged at 20,000 x *g* for 1 h at 4°C. To terminate the pepsin reaction, the supernatant obtained was dialyzed against 10 volumes of 0.02 M sodium phosphate buffer (pH 7.2) in a dialysis membrane with molecular weight cut off of 30 kDa for 24 h at 4°C with a change of solution every 4 h. The dialysate obtained was centrifuged at 20,000 x *g* for 1 h. The pellet obtained was dissolved in 10 volumes of 0.5 M acetic acid. The solution was further precipitated by addition of NaCl to a final concentration of 2.6 M in 0.05 M Tris-HCl (pH 7.5). The resultant precipitate was collected by centrifugation at 20,000 x *g* for 1 h and re-dissolved in 10 volumes of 0.5 M acetic acid. The solution was dialyzed and freeze-dried in the same manner with ASC preparation. Dry matter was referred to as pepsin-solubilized collagen (PSC).

Electrophoretic analysis

Protein pattern of collagen samples was analyzed using sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) according to the method of Laemmli (1970). Collagen samples (100 mg) were dissolved in 10 ml of 0.02 M sodium phosphate buffer (pH 7.2) containing 1% (w/v) SDS and 3.5 M urea. The sample mixtures were gently stirred at 4°C for 12 h to dissolve total proteins. Supernatants were collected after centrifuging at 3,000 x *g* for 3 min at 4°C. Solubilized collagen samples were mixed with the sample buffer (0.5 M Tris-HCl, pH 6.8 containing 4% (w/v) SDS, 20% (v/v) glycerol with and without 10% (v/v) β-ME.) at the ratio of 1:1 (v/v).

Samples were loaded on the PAGEL® -Compact precast gel (5% gel) and subjected to electrophoresis at a constant current of 20 mA per gel using a Compact-PAGE apparatus (Atto Co., Tokyo, Japan). After electrophoresis, gel was stained with 0.05% (w/v) Coomassie blue R-250 in 15% (v/v) methanol and 5% (v/v) acetic acid and destained with 30% (v/v) methanol and 10% (v/v) acetic acid. High molecular weight markers (Sigma Chemical Co., St. Louis, Mo, USA) were used to estimate the molecular weight of proteins. Calf skin acid-soluble type I collagen (Sigma Chemical Co., St. Louis, Mo, USA), porcine cartilage acid-soluble type II collagen, porcine skin acid-soluble type III collagen, and porcine placenta acid-soluble type V collagen (Wako Pure Chemical Industries, Ltd., Tokyo, Japan) were used as standard collagens.

Amino acid composition

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

Peptide mapping of collagen

Peptide mapping of collagen samples was performed according to the method of Saito *et al.* (2002) with a slight modification. The freeze-dried samples (0.2 mg) were dissolved in 0.1 ml of 0.1 M sodium phosphate, pH 7.2 containing 0.5% (w/v) SDS. After the addition of 10 μl of the same buffer containing 5 μg of *Staphylococcus aureus* V8 protease (EC 3.4.21.19, Sigma Chemical Co., St. Louis, Mo, USA) or 0.05 μg of lysyl endopeptidase from *Achromobacter lyticus* (EC 3.4.21.50; 4.5 AU/mg protein; Wako Pure Chemical Industries, Ltd., Tokyo, Japan) to collagen solutions, the reaction mixture was incubated at 37°C for 25 min or 5 min for V8 protease and lysyl endopeptidase, respectively. The reaction was terminated by heating in boiling water for 3 min. Peptides generated by the protease digestion were separated by SDS-PAGE using 7.5% gel. Peptide mapping of calf skin collagen acid-soluble type I was

conducted in the same manner and the peptide patterns were compared.

Collagen solubility test

The solubility of collagens was determined in 0.5 M acetic acid at various pHs and NaCl concentrations according to the method of Montero *et al.* (1991) with a slight modification. Collagen samples were dissolved in 0.5 M acetic acid with a gentle stirring at 4°C for 12 h to obtain a final concentration of 3 and 6 mg/ml.

To determine the effect of salt concentration on collagen solubility, 5 ml of collagen solutions (6 mg/ml) in 0.5 M acetic acid were mixed with 5 ml of cold NaCl in acetic acid with various concentrations of 0, 2, 4, 6, 8, 10, and 12% to obtain the final NaCl concentrations of 1, 2, 3, 4, 5, and 6%, respectively. The mixtures were stirred gently at 4°C for 30 min and centrifuged at 10,000 x g for 30 min at 4°C. Protein contents in the supernatants were determined by the method of Lowry *et al.* (1951) using bovine serum albumin as a protein standard. Relative solubility of collagen samples was calculated in comparison with that obtained at the NaCl concentration rendering the highest solubility.

To determine the effect of pH on collagen solubility, 8 ml of collagen solutions (3 mg/ml) were transferred to a centrifuge tube and the pH was adjusted with either 6 M NaOH or 6 M HCl to obtain a final pH ranging from 1 to 10. The volume of sample solutions was made up to 10 ml by distilled water, previously adjusted to the same pH of collagen sample solutions. The solutions were stirred gently for 30 min at 4°C and centrifuged at 10,000 x *g* for 30 min at 4°C. Protein contents in the supernatants were determined by the method of Lowry *et al.* (1951) using bovine serum albumin as a protein standard. Relative solubility of collagen samples was calculated as previously mentioned.

Thermal transition measurement

Collagen samples were prepared by the method described by Rochdi *et al.* (2000) with a slight modification. The freeze-dried collagen samples were rehydrated in deionized water or 0.05 M acetic acid solution with a sample per solution ratio of 1:40 (w/v). The mixtures were allowed to stand for 48 h at 4°C.

Thermal transition of collagen samples was measured using Perkin Elmer

Differential Scanning Calorimetry (DSC) (Model DSC-7, Norwalk, CT, USA). Temperature calibration was performed using the indium thermogram. The rehydrated samples (5-10 mg) were accurately weighed into aluminum pans, sealed, and scanned over the range of 20 to 50°C with a heating rate of 1°C/min. The ice water was used as a cooling medium and the system was equilibrated at 20°C for 5 min prior to the scan. The empty aluminum pan was used as the reference. The maximum transition temperature (T_{max}) was estimated from the maximum peak of DSC transition curve.

Statistical analysis

Data were subjected to analysis of variance (ANOVA). Comparison of means was carried out by Duncan's multiple-range test (Steel and Torrie, 1980). Analysis was performed using a SPSS package (SPSS 8.0 for window, SPSS Inc, Chicago, IL).

2.4 Results and Discussion

Isolation of ASC and PSC from bigeye snapper skin

Collagens from bigeye snapper skin were isolated into acid-solubilized collagen (ASC) and pepsin-solubilized collagen (PSC) with the yields of 6.4% and 1.1% (wet weight basis), respectively. Collagen in skin was solubilized to a high extent by 0.5 M acetic acid extraction. Further solubilization of remaining residues was achieved by limited pepsin digestion. The result suggested that the covalently cross-linking at the telopeptide region of collagen molecules through the condensation of aldehyde groups as well as the inter-molecular cross-linked molecules were not solubilized by acid extraction. These cross-linking molecules generally caused the decrease in solubility of collagen (Foegeding *et al.*, 1996; Belitz and Grosch, 1999). With the limited pepsin digestion, the cross-linked molecules at the telopeptide region were cleaved without damaging integrity of the triple helix. Nevertheless, the solubilized collagen with the predominant monomeric molecules could be obtained (Hickman *et al.*, 2000). From the result, the major fraction of collagen from bigeye snapper skin was ASC (85%; based on extractable collagen weight) and a lower content of PSC was found (15%). It was suggested that high inter-molecular cross-linked collagen molecule was present at a low content in the skin of bigeye snapper.

The high content of ASC fraction in bigeye snapper skin was in accordance with those reported in hake skin (*Merluccius hubbsi*) (85%; based on extractable collagen) (Ciarlo *et al.*, 1997) and trout skin (*Salmo irideus* Gibb) (95%; based on extractable collagen) (Montero *et al.*, 1991).

SDS-polyacrylamide gel electrophoresis (SDS-PAGE) patterns of ASC and PSC

The protein patterns of ASC and PSC from bigeye snapper skin under reducing and non-reducing conditions are shown in Figure 7. Generally, no differences in protein patterns of ASC or PSC under both conditions were observed. The result indicated that both collagen fractions contained no disulfide bond. Mizuta *et al.* (1994) reported that protein patterns of squid collagen were not changed in presence of β-ME. Both ASC and PSC were composed of α_1- and α_2-chains as the major constituents. High-molecular-weight components including β- and γ-components as well as their cross-linked molecules were also observed, especially in ASC. From the result, a high content of β-component was found, indicating the presence of cross-linking of collagen molecules. For both ASC and PSC, α_1-chain band intensity was 2-fold higher than that of α_2-chain. The patterns were also similar to the standard collagen type I from calf skin (Lane 2). These results suggested that the major collagen in both ASC and PSC extracted from bigeye snapper skin belonged to type I. This observation was in accordance with those previously reported in hake skin collagen (Ciarlo *et al.*, 1997; Montero *et al.*, 1990), trout (Montero *et al.*, 1990), Japanese sea-bass, and bullhead shark (Nagai and Suzuki, 2000a). Type I collagen consists of two α_1- and one α_2-chain (Wong, 1989). However, α_3-chain might be present in the collagens tested. If present, it could not be separated under the electrophoretic conditions employed because α_3-chain migrates electrophoretically to the same position as α_1-chain (Kimura, 1992; Nagai and Suzuki, 2000a; Ciarlo *et al.*, 1997). Apart from α-chains in both ASC and PSC, the high-molecular-weight components appeared with varying content, depending on solubilization process. The cross-links in collagen increase with animal age (Foegeding *et al.*, 1996) and the starving fish consists of collagen with a greater degree of cross-linking than do fish that are well fed (Sikorski *et al.*, 1990; Love *et al.*, 1976). However, the rate of cross-linking of collagen in fish skins is extremely slow (Cohen-Solal *et al.*, 1981) and the highly cross-linked

molecule is not generally found (Foegeding *et al.*, 1996). From the result, the major collagens in ASC and PSC extracted from bigeye snapper skin were shown to be heterotrimers $(\alpha 1)_2\alpha 2$, which contained no disulfide bond.

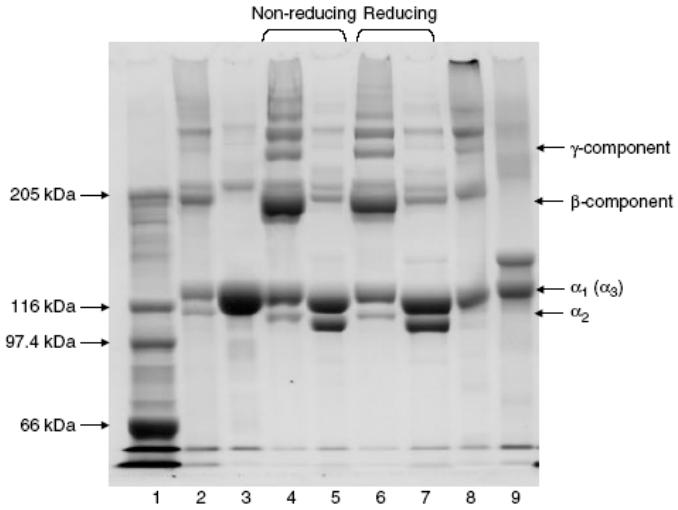

Figure 7. Protein patterns of ASC and PSC from bigeye snapper skin under reducing and non-reducing conditions. Lane 1: high molecular-weight protein markers; lanes 2, 3, 8 and 9, collagen type I, II, III and V, respectively; lanes 4 and 5, ASC and PSC under non-reducing conditions; lanes 6 and 7, ASC and PSC under reducing conditions.

Amino acid composition

The amino acid composition of ASC and PSC is shown in Table 4. Among all amino acids of ASC and PSC, glycine was found to be the dominant amino acid accounting about 23.7 and 27.2% of total amino acids, respectively. Both collagen fractions, ASC and PSC, also contained a high content of hydroxyproline (87 and 80 residues per 1,000 residues), hydroxylysine (12 and 13 residues per 1,000 residues), and proline (124 and 107 residues per 1,000 residues). Both ASC and PSC showed the similar amino acid composition. Generally, glycine distributes uniformly at every third position throughout most of collagen molecules (Foegeding *et al.*, 1996; Belitz and Grosch, 1999; Wong, 1989). The numbers of imino acids, proline and hydroxyproline, in ASC and PSC were 211 and 187 residues per 1,000 residues, respectively. The content of imino acid from bigeye snapper skin ASC was similar to that observed in calf skin collagen

(215 residues per 1,000 residues) (Li *et al.*, 2004). These amino acids were found to contribute to stability of collagen fibers and denaturation temperature (Ikoma *et al.*, 2003). The occurrence of hydroxyproline and hydroxylysine along the collagen molecule is due to the oxidation of hydroxylated residue of proline and lysine which was catalyzed by proline hydroxylase to form hydroxyproline and hydroxylysine, respectively (Foegeding *et al.*, 1996; Wong, 1989). Imino acids in some fish skin collagen, such as carp, common mackerel, hake, trout, ocellate puffer, South American lungfish, longnose gar, eagle ray, white sturgeon, and frilled shark ranged from 158 to 198 residues per 1,000 residues (Kimura, 1983; 1992; Nagai *et al.*, 2002; Montero *et al.*, 1990). As shown in this study and previous reports from other species, hydroxyproline content can vary with species and their habitat (Foegeding *et al.*, 1996; Sikorski *et al.*, 1990). This might contribute to the differences in properties of collagens among different species.

Table 4. Amino acid compositions of ASC and PSC from bigeye snapper skin (residues per 1000 total amino acid residues)

Amino acids	ASC	PSC
Hydroxyproline	87	80
Aspartic acid	53	55
Threonine	30	32
Serine	38	41
Glutamic acid	81	78
Proline	124	107
Glycine	237	272
Alanine	138	130
Valine	22	24
Methionine	16	13
Isoleucine	8	10
Leucine	25	27
Tyrosine	5	4
Phenylalanine	16	15
Hydroxylysine	12	13
Lysine	33	31
Histidine	10	9
Arginine	65	59
Total	1000	1000

Peptide mapping of collagen

Peptide maps of ASC and PSC digested by lysyl endopeptidase and V8 protease are shown in Figure 8. The decreases in band intensity of α_1- and α_2-chains as well as high-molecular-weight cross-links, γ- and β-components were observed with ASC and PSC after the limited digestion by V8 protease (Lanes 5-7) and lysyl endopeptidase (Lanes 8-10) with the concomitant increase in small MW peptide fragments. With the digestion by V8 protease, the band intensity of β-component and higher cross-linked molecules of calf skin collagen type I was slightly decreased and the major fragment with MW of 142 kDa was observed. For ASC and PSC, high MW cross-links were markedly degraded after digestion with V8 protease. The result suggested that high MW cross-links from calf skin collagen type I were more resistant to hydrolysis by V8 protease than ASC and PSC from bigeye snapper skin. The major degradation peptides appeared at MW of 148 and 135 kDa for ASC and 150 and 140 kDa for PSC. Both α-chains, α_1 and α_2, from calf skin collagen type I, ASC and PSC were hydrolyzed to some extent. After digestion, low MW peptide fragments with MW of 101 and 38 kDa were found with calf skin collagen type I, while peptide fragments with MW of 96, 84, 61, 38, and 34.6 kDa were generated. Additional peptide fragments with MW of 73 kDa was observed for ASC and those with MW of 71 and 58 kDa were obtained for PSC.

For peptide mapping of collagens digested by lysyl endopeptidase, all protein were more hydrolyzed, compared to the hydrolysis with V8 protease as evidenced by the lower original band intensity of each component remained with the concomitant larger increase in lower MW peptide fragments. β-component (175 kDa) and the higher cross-linked molecules of calf skin collagen, ASC, and PSC were susceptible to hydrolysis by lysyl endopeptidase. These components almost disappeared and peptide fragments with MW of 164, 146, and 139 kDa were generated. Peptide with MW of 153 kDa was only found in ASC and PSC after digestion but not in calf skin collagen. Both α-chains in calf skin collagen and ASC mostly disappeared after digestion, while those of PSC still remained to some extent. The smaller peptide fragments with MW of 105, 97, and 91 kDa were produced in calf skin collagen, ASC, and PSC. Although the similar MW peptide fragments were generated among all collagens, some differences in peptide fragments were also found. Fragments with MW of 71 and 33 kDa were only obtained in hydrolyzed calf

skin collagen type I. Generally, similar peptide patterns between ASC and PSC were observed. The same fragments with MW of 87, 76, 68, 54, 46, and 30 kDa were generated in both ASC and PSC. However, only peptide fragments with MW of 48, 38, 36, and 35 kDa were observed in ASC whereas that with MW of 32.0 kDa was only found in PSC. Nagai *et al.* (2002) reported that the peptide mapping of ASC and PSC from ocellate puffer fish skin hydrolyzed by lysyl endopeptidase was similar.

The differences in peptide fragments between different collagens generated by lysyl endopeptidase and V8 protease digestion suggested that there might be some differences in their primary structure (α-helix strand) (Nagai *et al.*, 2002; Yoshinaka *et al.*, 1991; Omura *et al.*, 1996). Peptide mapping was reported to differ among sources and species (Nagai *et al.*, 2002; Mizuta *et al.*, 1999). Therefore, ASC and PSC from bigeye snapper might be different in terms of domain or cross-links and were totally different from calf skin collagen in sequence and composition of amino acids.

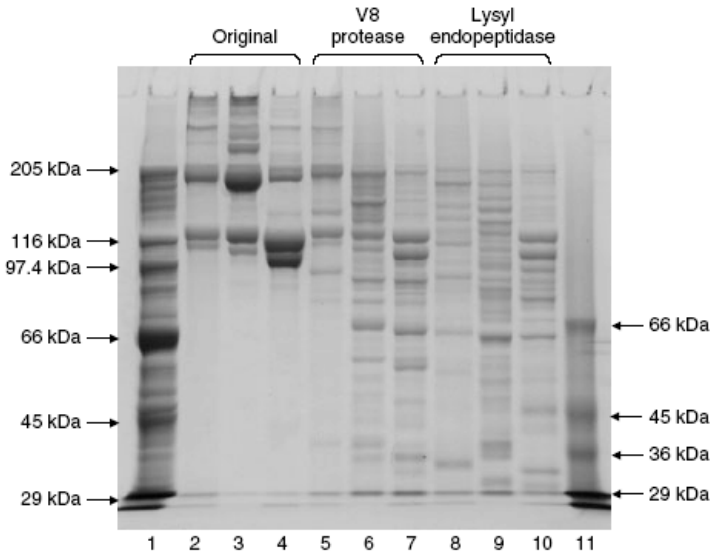

Figure 8. Peptide maps of ASC and PSC from bigeye snapper skin digested by V8 protease and lysyl endopeptidase. Lanes 1 and 11, high- and low-molecular-weight protein markers, respectively; lanes 2, 3 and 4, collagen type I, ASC and PSC; lanes 5, 6 and 7, peptide fragments of collagen type I, ASC and PSC with V8 protease digestion, respectively; lanes 8, 9 and 10, peptide fragments of collagen type I, ASC and PSC with lysyl endopeptidase digestion, respectively.

Effect of salt concentration on collagen solubility

The effect of NaCl on collagen solubility is shown in Figure 9. Solubility of both ASC and PSC in 0.5 M acetic acid decreased gradually with increasing NaCl concentration up to 3% ($P<0.05$). Sharp decrease in solubility was observed with NaCl concentration above 3% ($P<0.05$). Slight decrease in solubility was also observed with further increase in NaCl concentration (between 5 and 6%). Montero *et al.* (1991; 1999) reported that solubility of collagen from trout and hake skins in acetic acid solution decreased as NaCl concentration increased. The decrease in collagen solubility in presence of high NaCl concentration might be owing to salting-out effect. When ionic strength increased, the decrease in collagen solubility was possibly caused by an enhanced hydrophobic-hydrophobic interaction, and the competing of ionic salts for water, leading to the induced protein precipitation (Damodaran, 1996; Vojdani, 1996). From the result, PSC showed slightly greater solubility than ASC at the same NaCl concentration tested. Greater solubility of PSC might be due to the proteolytic action of pepsin. Additionally, some differences in compositions and conformation between both collagen fractions might result in such a difference. However, both collagen fractions were still soluble in presence of NaCl up to 3%.

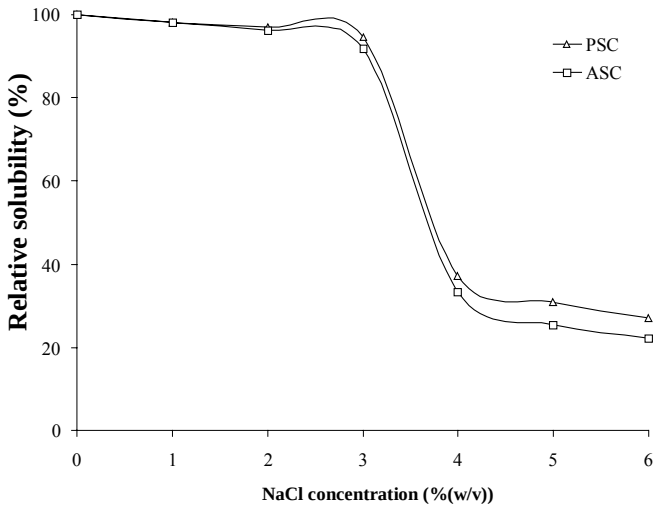

Figure 9. Solubility of ASC and PSC from bigeye snapper skin in 0.5 M acetic acid at different NaCl concentrations.

Effect of pHs on collagen solubility

The effect of pH on the solubility of ASC and PSC in 0.5 M acetic acid is shown in Figure 10. The highest solubility of ASC and PSC was observed at pH 4 and 5, respectively ($P<0.05$). In general, ASC and PSC were solubilized to a greater extent in the acidic pHs. The drastic decreases in solubility were found for both ASC and PSC fractions with the further increases in pH up to 6 and 7, respectively. At alkaline pH ranges, solubility slightly increased with an increasing pH value up to 10. From the result, PSC generally had higher solubility than ASC at all pHs tested excepted at the pH maximum for ASC, suggesting the lower degree of molecular cross-linking of PSC fraction, or the predominant weaker bonds than ASC (Montero *et al.*, 1990). This result was in coincidental with the higher contents of cross-linked molecules in ASC appeared on SDS-PAGE, compared to that in PSC (Figure 7). The variation in solubility of proteins with pH might be due to the differences in isoelectric point. As the pH is lower or higher than pI, the repulsion forces between charged residues of protein molecules are greater and the solubility of protein is increased (Damodaran, 1996). Conversely, hydrophobic-hydrophobic interaction is increased and the total net charges of protein molecules are zero, thereby causing protein precipitation and aggregation at pI (Wong, 1989; Lin *et al.*, 2002).

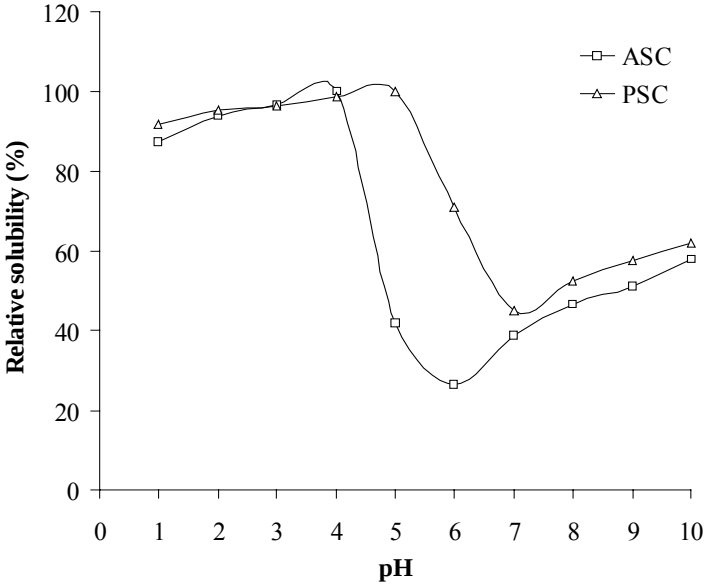

Figure 10. Solubility of ASC and PSC from bigeye snapper skin in 0.5 M acetic acid at different pHs.

Thermal stability of collagen

Thermal transitions of ASC and PSC in deionized water and 0.05 M acetic acid are depicted in Fig 5. The endothermic peaks of ASC and PSC were observed with the T_{max} of 30.37 and 30.87°C, respectively, in deionized water and at 28.85 and 29.38°C, respectively, in 0.05 M acetic acid. From the result, similar T_{max} between ASC and PSC in deionized water was observed, suggesting that both fractions had the similar denaturation temperature. Hickman *et al.* (2000) reported that pepsin cleaved the telopeptide region containing the intermolecular cross-linked without damaging the integrity of triple helix. As a consequence, triple helix structure was still predominant in both ASC and PSC, resulting in the similar thermal characteristic between both fractions. However, denaturation temperature of ASC and PSC from bigeye snapper was much lower than mammalian collagen (41°C) (Burjandze, 1979; Komsa-Penkova *et al.*, 1999). Relationship between hydroxyproline content and denaturation temperature has been established. Collagen containing the lower amount of hydroxyproline content denatures at the lower temperature than the larger does (Foegeding *et al.*, 1996). Moreover, hydroxyproline plays an important role in the stabilization of collagen triple-helix structure due to its hydrogen bonding ability through its -OH group (Foegeding *et al.*, 1996; Burjandze, 1979). In presence of 0.05 M acetic acid, the peak was shifted to lower temperature. Thus, acetic acid might change the conformation of collagen in the fashion which was more prone to denaturation. The inter-chain hydrogen bonds stabilizing the collagen triple-helix structure were partially cleaved by acetic acid (Gustavson, 1956). The denaturation temperature of skin collagen from bigeye snapper showed higher temperature than other previously reported from some cold-water fish species. Thermal transition temperatures of skin collagen have been reported for hake (10°C) (Ciarlo *et al.*, 1997), Alaska pollack (16.8°C) (Kimura and Ohno, 1987), Japanese sea-bass (26.5°C), chub mackerel (25.6°C), bullhead shark (25.0°C) (Nagai and Suzuki, 2000a), ocellate puffer (28°C) (Nagai *et al.*, 2002) and Baltic cod (15°C) (Sadowska *et al.*, 2003). The higher denaturation temperature was attributed to the higher imino acid content of land animal collagen than tropical, and temperate fish (Kimura, 1992; Li *et al.*, 2004; Gustavson, 1955). Furthermore, the environmental and body temperature among living habitat has been reported to correlate with denaturation temperature of collagen (Kimura and Ohno, 1987; Eastoe, 1957; Rigby, 1968; Kimura and Tanaka, 1986; Sivakumar, 2000). Thus, collagen from bigeye snapper

skin had the high thermal stability, which might be associated with the different properties of collagen from other species.

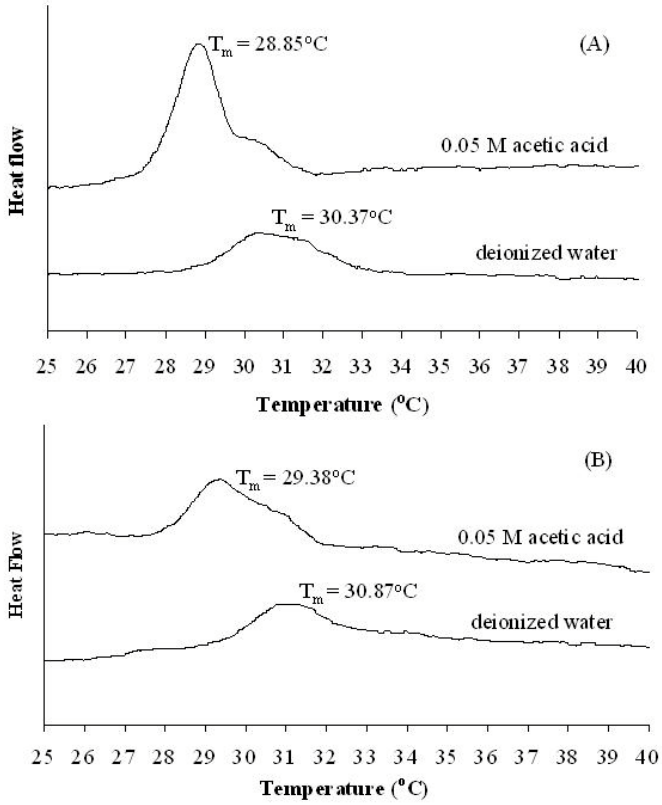

Figure 11. Thermograms of collagen ASC (A) and PSC (B) from bigeye snapper skin rehydrated in 0.05 M acetic acid and deionized water.