

CHAPTER 3

ISOLATION AND CHARACTERISATION OF ACID AND PEPsin-SOLUBILIZED COLLAGENS FROM THE SKIN OF BROWNSTRIPE RED SNAPPER (*LUTJANUS VITTA*)

3.1 Abstract

Acid-solubilized collagen (ASC) and pepsin-solubilized collagen (PSC) were successfully extracted from the skin of brownstripe red snapper (*Lutjanus vitta*) with the yields of 9% and 4.7%, respectively, on the basis of wet weight. Both ASC and PSC comprised two different α chains ($\alpha 1$ and $\alpha 2$), and were characterized to be type I with no disulfide bond. PSC contained a lower content of high molecular weight cross-links, compared with ASC. Peptide maps of ASC and PSC hydrolysed by V8 protease and lysyl endopeptidase showed some differences in peptide patterns between the two fractions and were totally different from those of calf skin collagen type I, suggesting differences in amino acid sequences and collagen conformation. Maximum solubility in 0.5 M acetic acid was observed at pH 3 and pH 4 for ASC and PSC, respectively. A sharp decrease in solubility was observed in the presence of NaCl above 2% and 3% (w/v) for ASC and PSC, respectively. T_{max} of both collagen fractions were similar and shifted to a lower value in the presence of acetic acid, suggesting some changes in the collagen structure caused by acid induction.

3.2 Introduction

Collagen is an abundant protein in vertebrates and constitutes approximately 30 % of total animal protein (Muyonga *et al.*, 2004a). Tendon, skin, bone, vascular system and connective tissue sheaths surrounding muscle are the organs of animals that are mainly structured by collagen (Foegeding *et al.*, 1996). Furthermore collagen has been found in fish skin, bone and scale (Kimura, 1992; Nagai and Suzuki, 2000a; Nomura *et al.*, 1996; Ikoma *et al.*, 2003). Nineteen variants of collagen have been reported, named type I–XIX (Bailey *et al.*, 1998). So far, the main sources of collagen are limited to

based animal such as bovine or porcine skin and bone. However, the outbreak of bovine spongiform encephalopathy (BSE) and the foot-and-mouth disease (FMD) crisis has resulted in anxiety among users of collagen and collagen-derived products from land animal origin (Helcke, 2000). Additionally, collagen obtained from porcine 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 extraction.

The fish processing industry is one of an important income generators for Thailand. During processing, a large amount of wastes is generated. Fish solid wastes constitute 50 - 70% of the original raw material, depending on the processes used, types of products, etc. Those wastes have been of interest to use as high-protein human foods, instead of employing them as pet foods (Shahidi, 1994; Montero *et al.*, 1991). Among the value-added products derived from those wastes, collagen from skin, scale and bone has been increasingly of interest owing to its abundance. Collagen content (Acid-solubilized collagen; ASC) in fish skins of young and adult Nile perch were 63.1 and 58.7%, respectively (Muyonga *et al.*, 2004a), whereas those of Japanese sea-bass, chub mackerel and bullhead shark were 51.4, 49.8 and 50.1%, respectively (Nagai and Suzuki, 2000a). Collagen contents generally vary with fish species, age and season (Foegeding *et al.*, 1996; Nagai *et al.* 2002; Ciarlo *et al.*, 1997; Montero *et al.*, 1991). Type I collagen is the fibrous collagen and is found as the major type in fish waste materials including skin, bone, scale and fins of various fish species (Kimura, 1992; Nagai and Suzuki, 2000a; Nagai *et al.*, 2002; Sato *et al.*, 1989, Kimura, 1983, Ikoma *et al.*, 2003). Recently, biochemical properties of black drum and sheepshead seabream skin collagen which are subtropical fish have been characterized (Ogawa *et al.*, 2003). Nevertheless, collagen from different species and habitat 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 involving those used for surimi production, has been reported. Therefore, this investigation aimed to isolate and characterise acid and pepsin solubilized collagens from the skin of brownstripe red snapper (*Lutjanus vitta*) which is one of the main fish species used for surimi production in Thailand.

3.3 Materials and methods

Chemicals

β -mercaptoethanol (β ME), pepsin (EC 3.4.23.1) powdered; 750 U/mg dry matter, *Staphylococcus aureus* V8 protease (EC 3.4.21.19) and protein markers 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). *Achromobacter lyticus* Lysyl endopeptidase (EC 3.4.21.50) was from Wako Pure Chemical Industries, Ltd. (Tokyo, Japan).

Fish skin preparation

Brownstripe red snapper (*Lutjanus vitta*) with an average 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 at the dock in Songkhla, fish were stored in ice with a fish/ice ratio of 1:2 (w/w) and transported to the Department of Food Technology, Prince of Songkla University, Hat Yai. Fish were washed using 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/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/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/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 at 4°C. The

supernatants were collected and kept at 4°C. The precipitate was re-extracted in 0.5 M acetic acid with a sample/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 at 4°C. The supernatants obtained were combined with the first extract. The combined extracts were 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 at 4°C and then dissolved in 10 volumes of 0.5 M acetic acid. The solution obtained was dialysed 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 dialysed with 10 volumes of distilled water with the changes of water until neutral pH was obtained. The dialysate was freeze-dried and referred to as acid-solubilized collagen (ASC). Undissolved residue obtained after acid extraction was thoroughly rinsed with distilled water, suspended in 2 volumes of 0.5 M acetic acid and subjected to limit hydrolysis with 10% (w/v) pepsin (EC 3.4.23.1; powderised; 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 dialysed 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 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 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 at 4°C and re-dissolved in 10 volumes of 0.5 M acetic acid. The solution was dialysed with distilled water and freeze-dried in the same manner as for ASC preparation. Dry matter was referred to as pepsin-solubilized collagen (PSC).

Electrophoretic analysis

Protein patterns of collagen samples were analysed using sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) according to the method of Laemmli (1970). Collagen samples were dissolved in 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 using the sample/sample buffer 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/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., U.S.A.), 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 hydrolysed under reduced pressure in 4.0 M methanesulfonic acid containing 0.2% (v/v) 3-(2-aminoethyl)indole at 115°C for 24 h, and the hydrolysates were neutralised 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 analyser (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 μ l of the same buffer containing 5 μ g of *Staphylococcus aureus* V8 protease (EC 3.4.21.19, 800 unit/mg solid Sigma Chemical Co., St. Louis, Mo., USA) or 0.05 μ g 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

subjecting the reaction mixture to boiling water for 3 min. Peptides generated by the protease digestion were separated by SDS-PAGE using 7.5% gel. Peptide mapping of calf skin collagen acid-soluble type I was conducted in the same manner and the peptide maps were compared.

Collagen solubility test

The solubility of collagens was determined in 0.5 M acetic acid at various pH 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 the final concentrations of 3 and 6 mg/ml.

Effect of pHs on collagen solubility

Eight 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 with distilled water previously adjusted to the same pH of collagen sample solutions tested. 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 content in the supernatants was 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 pH rendering the highest solubility.

Effect of salt concentration on collagen solubility

Five 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 (0, 2, 4, 6, 8, 10, and 12% (w/v)) to obtain the final NaCl concentrations of 1, 2, 3, 4, 5, and 6% (w/v). 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 content in the supernatants was determined by the method of Lowry *et al.* (1951) and relative solubility was calculated in comparison with that found at the salt concentration exhibiting the highest solubility.

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 deionised water or 0.05 M acetic acid solution with a sample/solution ratio of 1:40 (w/v). The mixtures were allowed to stand for 2 days at 4°C.

The thermal transition of collagens 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).

3.4 Results and discussions

Isolation of ASC and PSC from brownstripe red snapper skin

Acid-solubilized collagen (ASC) and pepsin-solubilized collagen (PSC) were isolated from brownstripe red snapper skin with the yields of 9.0% and 4.7% (wet weight basis), respectively. The skin was not completely solubilized by 0.5 M acetic acid extraction. This result was in agreement with Jongjareonrak *et al.* (2005a) who reported the incomplete solubilisation of bigeye snapper skin in 0.5 M acetic acid. The result suggested that the collagen molecules in brownstripe red snapper skin were most likely cross-linked by covalent bonds through the condensation of aldehyde groups at the

telo peptide region as well as the inter-molecular cross-linking, leading to the decrease in solubility of collagen (Foegeding *et al.*, 1996; Burghagen, 1999). With further limited pepsin digestion, the cross-linked molecules at the telopeptide region were cleaved without damaging the integrity of the triple helix. Therefore, the collagen with the predominant monomeric molecules could be solubilized in the presence of acid (Hickman *et al.*, 2000). From the result, the major fraction of collagen from brownstripe red snapper skin was ASC (66% based on extractable collagen weight) and a lower content of PSC was found (34%). The greater content of ASC fraction in brownstripe red snapper skin was in accordance with those reported in bigeye snapper (*Priacanthus macracanthus*) skin (85%) (Jongjareonrak *et al.*, 2005a) and hake (*Merluccius hubbsi*) skin (85%) (Ciarlo *et al.*, 1997). However, the PSC fraction in brownstripe red snapper skin was 2-fold higher than those obtained from those two species. It is suggested that collagen with higher inter-molecular cross-link was present to a greater extent in the skin of brownstripe red snapper, compared with those found in bigeye snapper and hake skin.

SDS-polyacrylamide gel electrophoresis (SDS-PAGE)

The electrophoretic patterns of ASC and PSC were analysed in the presence and absence of β ME (Figure 12). Generally, no differences in the electrophoretic patterns of ASC or PSC with and without β ME were observed, indicating that both collagen fractions contained no disulfide bond. Montero *et al.* (1990) reported that a similar electrophoretic diagram of collagen from the skin of both hake and trout was observed in presence and absence of β ME. From the result, $\alpha 1$ - and $\alpha 2$ - chains were found as the major constituents for both ASC and PSC. High-molecular-weight components (High MW components) including β - and γ - components as well as their cross-linked molecules were also observed in both fractions.

High MW cross-linked molecules in collagen increase with animal age (Foegeding *et al.*, 1996) and starving fish consists of greater cross-linked collagen than those that are well fed (Sikorski *et al.*, 1990; Love *et al.*, 1976). However, the cross-linking rate of collagen in fish skins is extremely slow and the highly cross-linked molecule is rarely found (Cohen-Solal *et al.*, 1981). High MW cross-linked molecules in ASC were possibly digested, as evidenced by the lower density of β - and γ - component bands

with a concomitant increase in band intensity of α -chains in PSC fraction. Pepsin cleaves the cross-link containing telopeptide, and β -chain is concomitantly converted to two α -chains (Sato *et al.*, 2000). The band intensity of $\alpha 1$ -chain was 2-fold higher than that of $\alpha 2$ -chain for both ASC and PSC. The $\alpha 1$ - and $\alpha 2$ - chain pattern was similar to that of standard collagen type I from calf skin (Lane 2). It is suggested that type I collagen is the major collagen in both ASC and PSC from brownstripe red snapper skin. This observation is in agreement with finding reported for skin collagen from hake (Ciarlo *et al.*, 1997; Montero *et al.*, 1990), trout (Montero *et al.*, 1990), Nile perch (Muyonga *et al.*, 2004a), blackdrum and sheephead seabream (Ogawa *et al.*, 2003) and bigeye snapper (Kittiphattanabawon *et al.*, 2005). Type I collagen consists of two $\alpha 1$ - and one $\alpha 2$ -chain as the major component ($[\alpha 1]_2\alpha 2$). Since the $\alpha 3$ -chain has a molecular mass indistinguishable from $\alpha 1$ and as it cannot be separated from $\alpha 1$ under the electrophoretic conditions employed, the co-presence of $\alpha 3$ with $\alpha 1$ might be possible.

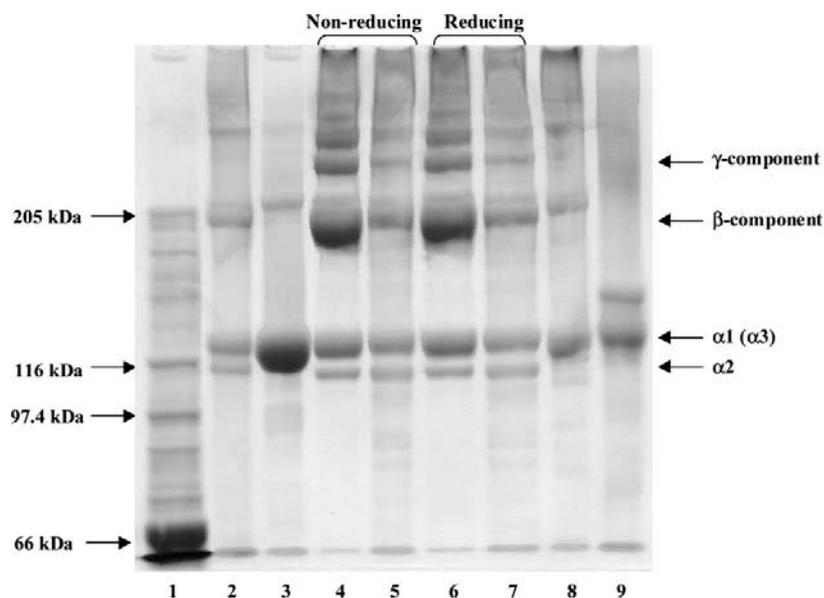


Figure 12. Protein pattern of ASC and PSC from Brownstripe red snapper skin under reducing and non-reducing conditions. Lane 1: high MW protein markers; lanes 2, 3, 8, and 9: collagen types I, II, III, and V, respectively; lanes 4 and 5: ASC and PSC under non-reducing conditions; lanes 6 and 7, ASC and PSC under reducing conditions.

Amino acid composition

The amino acid composition of ASC and PSC is expressed as residues per 1,000 total amino acid residues and is shown in Table 5. Generally, ASC and PSC extracted from brownstripe red snapper skin had similar amino acid profiles. From the results, ASC and PSC were rich in glycine (25.2% and 23.5%), alanine (14.3% and 14.2%), and proline (13.1% and 13.5%). In general, glycine represents uniformly at every third residue throughout most of collagen molecules except for that first 14 amino acid from the N-terminus and the first 10 from the C-terminus (Burghagen, 1999; Foegeding *et al.*, 1996; Wong, 1989). Both ASC and PSC fractions consisted of proline, hydroxyproline and hydroxylysine, which are the unique amino acids found in collagen. The amounts of imino acids, proline and hydroxyproline, in ASC and PSC were 212 and 221 residues per 1000 residues, respectively. The contents of imino acids in ASC and PSC from brownstripe red snapper skin were relatively high and were similar to that of ASC from calf skin (215 residues per 1000 residues) (Herbage *et al.*, 1977). Imino acids contribute to the denaturation temperature and the stability of helix structure of collagen (Ikoma *et al.*, 2003). ASC and PSC contained high levels of hydroxyproline and hydroxylysine and were similar to ASC from calf skin reported by Herbage *et al.* (1977). This result suggested a high degree of oxidation of hydroxylated residues of proline and lysine in collagens from brownstripe red snapper skin. The formation of hydroxyproline and hydroxylysine was catalysed by proline hydroxylase and lysine hydroxylase, respectively (Foegeding *et al.*, 1996; Wong, 1989). Imino acid contents of collagen from brownstripe red snapper skin were relatively high, compared with those reported for collagen from skins of carp, hake, trout, ocellate puffer, black drum, sheephead, bigeye snapper, which contained imino acids ranging from 158 to 211 residues per 1000 residues (Kimura, 1992; Montero *et al.*, 1990; Nagai *et al.*, 2002; Ogawa *et al.*, 2003; Kittiphattanabawon *et al.*, 2005; Jongjareonrak *et al.*, 2005a). Proline and hydroxyproline contents vary with species and their living habitat (Foegeding *et al.*, 1996; Love *et al.*, 1976). The pyrrolidine rings of proline and hydroxyproline impose restrictions on the conformation of the polypeptide chain and help to strengthen the triple helix (Wong, 1989). Thus, collagen from brownstripe red snapper might have a different molecular conformation from that of other species owing to the high content of imino acids.

Table 5. Amino acid composition of ASC and PSC from Brownstripe red snapper skin (residues per 1000 total amino acid residues)

Amino acids	ASC	PSC
Hydroxyproline	81	86
Aspartic acid	50	49
Threonine	29	30
Serine	37	39
Glutamic acid	81	79
Proline	131	135
Glycine	252	235
Alanine	143	142
Valine	18	17
Methionine	15	14
Isoleucine	7	8
Leucine	24	24
Tyrosine	4	2
Phenylalanine	15	16
Hydroxylysine	9	15
Lysine	33	34
Histidine	7	6
Arginine	65	68
Total	1000	1000

Peptide mapping of collagen

Peptide maps of collagens (ASC and PSC) digested by V8 protease and lysyl endopeptidase, in comparison with calf skin collagen type I, were revealed by SDS-PAGE (7.5% gel) as shown in Figure 13. Generally, band intensity of $\alpha 1$ - and $\alpha 2$ -chains as well as high MW cross-link, γ - and β - components of ASC, PSC and calf skin collagen type I decreased after the limited digestion by V8 protease (Lanes 5-7) and lysyl endopeptidase (Lanes 8-10). A concomitant increase in small peptides was also observed. Calf skin collagen type I digested by V8 protease (Lane 5) had a slight decrease in band

intensity of the α -, β -components, and high MW cross-linked molecules and the appearance of peptide fragments with MW of 154.7 kDa, 95.0 kDa, 83.6 kDa, and 38.6 kDa was noticeable. For ASC and PSC, the α -component and high MW cross-linked molecules were more hydrolysed after digestion with V8 protease. The results suggest that the α -component and high MW cross-linked molecules from calf skin collagen type I are more tolerant to hydrolysis by V8 protease than ASC and PSC from brownstripe red snapper skin. V8 protease shows a high specific preference to glutamic acid and aspartic acid residues of proteins (Vercaigne-Marko *et al.*, 2000). Due to the lower contents of glutamic acid and aspartic acid residues (75 and 45 residues per 1000 residues) in calf skin collagen (Herbage *et al.*, 1977), ASC (81 and 50 residues per 1000 residues) and PSC (79 and 49 residues per 1000 residues), which had the greater glutamic acid and aspartic acid contents, might be more susceptible to hydrolysis by V8 protease. From the result, ASC was more prone to hydrolysis than PSC. However, similar patterns of peptide fragments were observed. After hydrolysis, the α -component and high MW cross-linked molecules of ASC from the skin of brownstripe red snapper were degraded into small MW peptides ranging from 103.6 kDa to 34.0 kDa. In addition, peptide fragments with high MW ranging from 205 kDa to 116 kDa were also observed. For PSC, the α -component was hydrolysed to some extent, while high MW cross-linked molecules were totally digested and small MW peptides were also observed with molecular weights ranging from 103.6 kDa to 32.3 kDa.

For the peptide map of collagens digested by lysyl endopeptidase (Lanes 8-10), all collagens were more susceptible to hydrolysis by lysyl endopeptidase compared to V8 protease, as evidenced by the disappearance of the β -component (205 kDa) and the high MW cross-linked molecules with the concomitant increase in small MW fragments. This result was in accordance with the peptide mapping of collagens from skin and bone of bigeye snapper as reported by Kittiphattanabawon *et al.*, 2005. The α -component and the high MW cross-linked molecules of calf skin collagen type I and ASC from brownstripe red snapper skin mostly disappeared after hydrolysis, while the α -component of PSC still remained to some extent. The generated peptides from ASC and PSC after digestion had MWs ranging from 205 kDa to 30 kDa, while those of calf skin collagen were lesser in number of bands. The differences in peptide map between the different collagens generated by lysyl endopeptidase and V8 protease digestion suggests that there might be some

differences in their primary structure, especially the α -helix strand (Nagai *et al.*, 2002; Yoshinaka *et al.*, 1991; Omura *et al.*, 1996). Accessibility of susceptible bonds in collagen structure to proteinase might be different, causing the varying degrees of hydrolysis between ASC and PSC. Peptide maps of collagens were reported to differ among sources and species (Mizuta *et al.*, 1999). Thus, ASC and PSC from brownstripe red snapper skin might be different in terms of domain or cross-links and totally different from calf skin collagen type I in term of sequence and composition of amino acids.

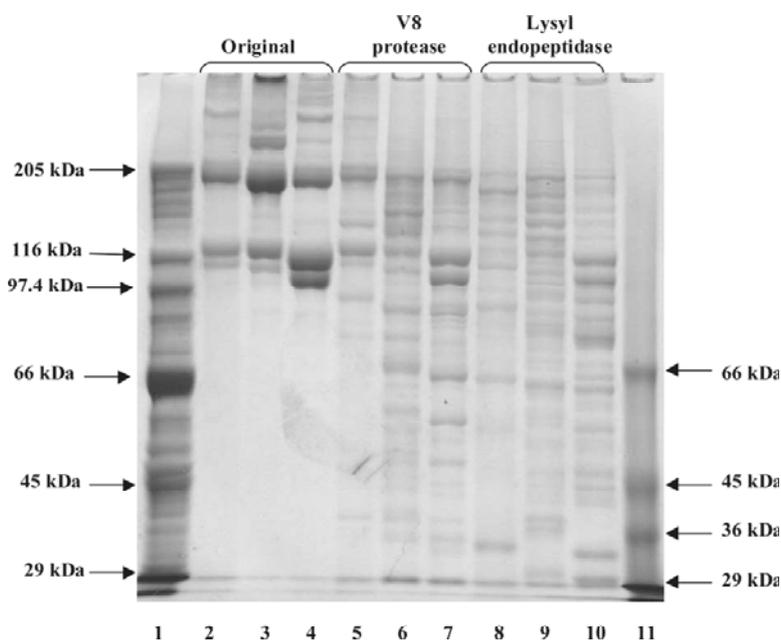


Figure 13. Peptide maps of ASC and PSC from Brownstripe red snapper skin digested by V8 protease and lysyl endopeptidase. Lanes 1 and 11: high and low MW protein markers, respectively; lanes 2, 3, and 4: collagen type I, ASC, and PSC; lanes 5, 6, and 7: peptide fragments of collagen type I, ASC, and 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 pHs on collagen solubility

The effect of pH on the solubility of ASC and PSC from brownstripe red snapper skin is shown in Figure 14. The solubilities of ASC and PSC reached maxima at

pH 3 and 4, respectively ($P < 0.05$). In general, both collagens were solubilized to a greater extent in acidic pH ranging from 1 to 4. Marked decreases in solubility were observed and reached the minimum when the pH was increased to 7 ($P < 0.05$). The further increases in pH up to 10 caused a slight increase in solubility. As the pH is lower or higher than pI, the net charge residues of protein molecules are greater and the solubility is increased by the repulsion force occurred between chains (Vojdani, 1996). In contrast, total net charges of protein molecules are zero and hydrophobic-hydrophobic interaction increases, thereby leading to the precipitation and aggregation at pI. It has been reported that collagen has the isoelectric points of pH 6–9 (Foegeding *et al.*, 1996). Thus the lowest solubility of ASC and PSC was obtained at pH around 7. This result was in accordance with the solubility of collagen from trout muscle and skin, which was lowest at pH 7 (Montero *et al.*, 1991). From the result, ASC was solubilized to a higher extent, compared with PSC at all pHs tested except at pH 2 and 3, suggesting a higher degree of molecular crosslinking of the ASC fraction, or the predominance of stronger bonds than PSC (Montero *et al.*, 1999). This was evidenced by the higher contents of high MW cross-link of ASC compared to that of PSC (Figure 12). The variation in solubility of collagens with pH has been reported for bigeye snapper skin and bone over the pH ranges of 1–10 (Kittiphattanabawon *et al.*, 2005).

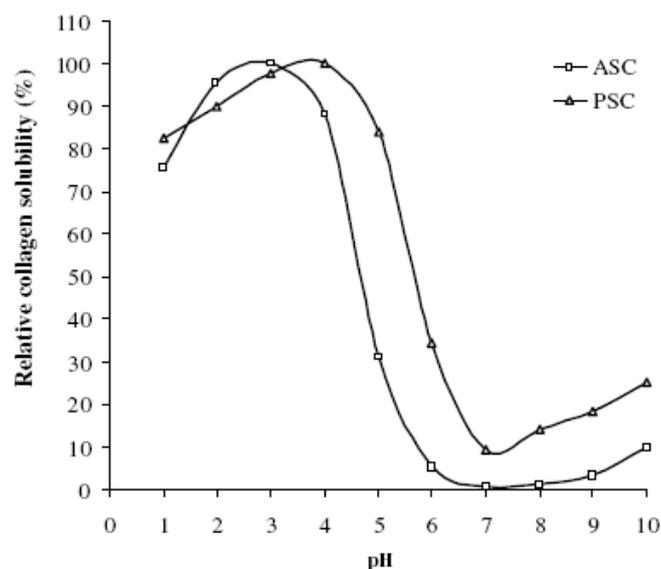


Figure 14. Solubility of ASC and PSC from Brownstripe red snapper skin in 0.5 M acetic acid at different pHs.

Effect of salt concentration on collagen solubility

The effect of NaCl on the solubility of ASC and PSC extracted from brownstripe red snapper skin is depicted in Figure 15. Solubility of ASC and PSC in 0.5 M acetic acid remained constant in the presence of NaCl concentration up to 2% ($P>0.05$). A drastic decrease in the solubility of ASC was observed at 3% NaCl or above ($P<0.05$). For PSC, a slight decrease in solubility was obtained in the presence of 3% NaCl. A sharp decrease in solubility was observed with 4% NaCl or above. The solubility of collagens from the skin of trout, hake, bigeye snapper (*Priacanthus tayenus*), and bigeye snapper (*Priacanthus macracanthus*) in acetic acid solution generally decreased with increasing NaCl concentration (Montero *et al.*, 1991; Montero *et al.*, 1999; Kittiphattanabawon *et al.*, 2005; Jongjareonrak *et al.*, 2005a). The decrease in solubility of collagens could be described by the salting out phenomenon which occurred at relatively low NaCl concentrations (Asghar and Henrickson, 1982). An increase in ionic strength causes a reduction in protein solubility by an enhanced hydrophobic-hydrophobic interaction between protein chains, and the competing for water of ionic salts, leading to the induced protein precipitation (Damodaran, 1996; Vojdani, 1996). From the result, similar behavior was found between both collagen fractions. However, PSC exhibited a greater solubility than ASC at NaCl concentration greater than 2%. A greater solubility of PSC could possibly be due to the partial hydrolysis of high MW cross-linked molecules by pepsin. In addition, the differences in compositions and molecular species between ASC and PSC fractions might result in such a different characteristic.

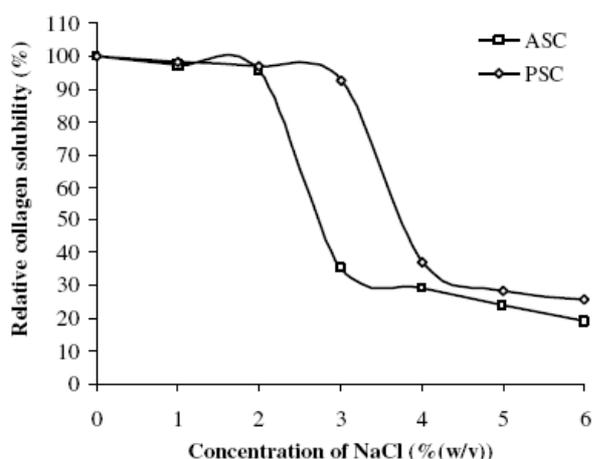


Figure 15. Solubility of ASC and PSC from Brownstripe red snapper skin in 0.5 M acetic acid with different NaCl concentrations.

Thermal stability of collagen

Thermal transitions of ASC and PSC from brownstripe red snapper skin rehydrated in deionised water and 0.05 M acetic acid are depicted in Figure 16. ASC and PSC showed the transition curves with the maximum temperatures (T_{\max}) of 31.52 and 31.02°C, respectively, in deionised water and at 30.52 and 30.46°C, respectively, in 0.05 M acetic acid. T_{\max} of ASC was similar to that of PSC in both media, suggesting no differences in the denaturation temperature between both fractions. The triple helix structure was still predominant in PSC fraction when the skin material was limited to digestion by pepsin (Hickman *et al.*, 2000). T_{\max} of ASC and PSC from brownstripe red snapper skin was much lower when compared with that of mammalian collagen, such as calf skin collagen (37°C) (Ogawa *et al.*, 2003). Imino acid contents (proline and hydroxyproline) show the direct positive correlation to the thermal stability of protein via hydrogen bond (Sikorski *et al.*, 1984). Therefore, calf skin collagen which contains a higher hydroxyproline content (94 residues per 1000 residues) than ASC and PSC (81 and 86 residues per 1000 residues, respectively), denatures at higher temperature. Denaturation temperatures of collagens from brownstripe red snapper skin were higher than those of collagen from other species such as hake (10°C), Baltic cod (15°C), Alaska pollack (16.8°C), bullhead shark (25°C), Japanese seabass (26.5°C), and ocellate puffer (28°C) (Ciarlo *et al.*, 1997; Sadowska *et al.*, 2003; Kimura and Ohno, 1987; Nagai *et al.*, 2000; Nagai *et al.*, 2002). The higher denaturation temperature of ASC and PSC from the skin of brownstripe red snapper, which is a tropical fish possibly, resulted from the higher content of imino acids (Table 5) than those of temperate fish.

The denaturation temperature of both collagens shifted to a lower temperature in the presence of 0.05 M acetic acid, when compared with those rehydrated in distilled water. Intramolecular hydrogen bonds which plays the important role in stabilising the triple helix structure (Ramachandran, 1988) of collagen were cleaved by acetic acid (Gustavson, 1956). Therefore, the helix-coil structure was destroyed, resulting in a decrease in the transition temperature of collagens rehydrated in 0.05 M acetic acid as observed by the lower T_{\max} of both ASC and PSC.

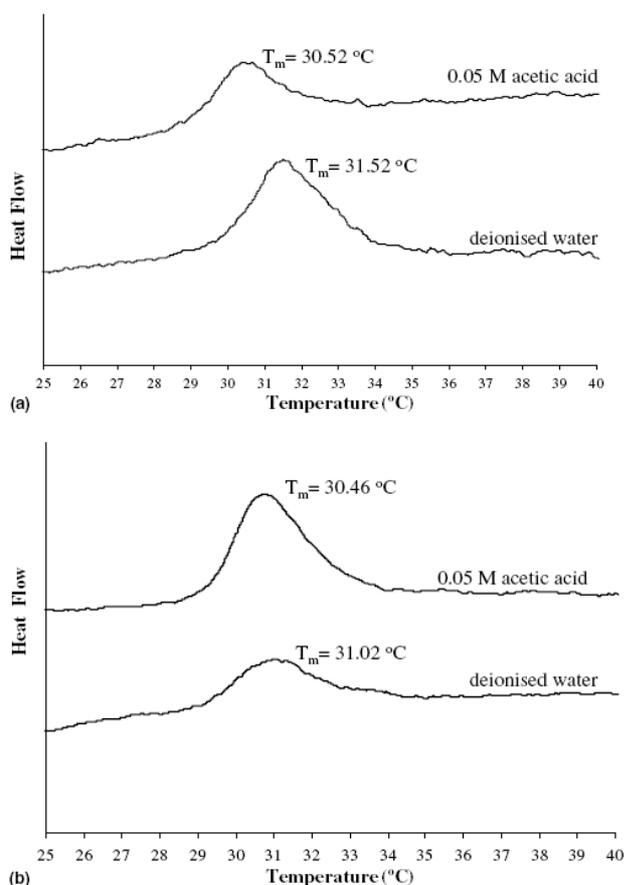


Figure 16. Thermograms of ASC (a) and PSC (b) from Brownstripe red snapper skin rehydrated in 0.05 M acetic acid and deionised water.

3.5 Conclusion

ASC and PSC from the skin of brownstripe red snapper were isolated with yields of 9 and 4.7%, respectively. The collagens were characterized to be type I without disulfide bond. ASC exhibited some differences in amino acids profiles, protein patterns, and sequence in their primary structure, compared with PSC and was totally different from calf skin collagen. Denaturation temperature of collagens was similar between ASC and PSC and was relatively higher than that of temperate fish. ASC and PSC were soluble in acidic pH ranges and lost the solubility with increasing salt concentrations.