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Isolation and characterization of collagen from bigeye snapper (*Priacanthus macracanthus*) skin

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Abstract: Acid-solubilized collagen (ASC) and pepsin-solubilized collagen (PSC) were isolated from the skin of bigeye snapper (*Priacanthus macracanthus*) with yields of 64 and 11 g kg^{-1} wet weight, respectively. Both ASC and PSC were characterized as type I collagens with no disulfide bonds. 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. A sharp decrease in solubility of both collagens in acetic acid was found with NaCl concentration above 30 g l^{-1}. Thermal transitions of ASC and PSC in deionized water were observed with T_{max} of 30.37 and 30.87 °C, respectively, and were lowered in the presence of acetic acid (0.05 mol kg^{-1} solution). Therefore, ASC was a major fraction in bigeye snapper skin and it exhibited some different characteristics to PSC.
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Keywords: collagen; bigeye snapper; skin; acid-solubilized collagen; pepsin-solubilized collagen; solubility

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.[1] Furthermore, collagen has been found in fish skin, bone and scale[2–5] as well as in shellfish tissue.[6–9] Collagen and gelatin have been widely used in food industries as ingredients to improve the elasticity, consistency and stability of foods. Collagen can be used for encapsulation and edible film formation, making it of interest to the pharmaceutical, biomaterial-based packaging and photographic industries.[10–14] Collagen and gelatin of land animal origin such as bovine and porcine skins and bones have mainly been used.[15] 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.[16] Therefore, alternative sources, such as fish processing waste including skin, bone or scale, have received increasing attention for collagen and gelatin extraction.

Surimi is one of several important income generators for Thailand, and is exported to several countries. The fish species used for its production include mainly threadfin bream (*Nemipterus* spp), bigeye snapper (*Priacanthus* spp), croaker (*Pennahia* and *Johnius* spp) and lizardfish (*Saurida* spp).[17] During processing a large amount of waste is generated. Fish solid wastes constitute 500–700 g kg^{-1} of the original raw material, depending on the method of meat extraction from the carcass.[18] This waste is an excellent raw material for the preparation of high-protein foods, besides helping to eliminate its harmful environmental effects.[19] About 300 g kg^{-1} of such wastes are skin and bone with high collagen content.[20] Nagai and Suzuki[3] reported that the collagen contents in fish skin waste from Japanese sea bass, chub mackerel and bullhead shark were 514, 498 and 501 g kg^{-1}, respectively, on the basis of lyophilized dry weight. Collagen contents vary, depending on fish species.[9,21,22] Type I collagen has been found as the major collagen in the skin, bone and fins of various fish species.[2,3,9,23,24] Recently, collagen subunits from shark skin and sea cucumber[25,26] have been characterized. Nevertheless,

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collagen from different species and habitats might be different in terms of molecular composition and properties.[1] So far, 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.

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 0.22–0.25 m were caught from Songkhla coast along the Gulf of Thailand, stored in ice and off-loaded after 24–36 h from 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, cut into small pieces (0.5 × 0.5 cm) and kept on ice prior to collagen extraction.

Skin collagen preparation

The collagen was extracted according to the method of Nagai and Suzuki[3] with a slight modification. All processes were carried out at 4 °C. Skin was soaked in 0.1 mol kg^{-1} NaOH (30 ml g^{-1}) for 24 h with gentle stirring. The solution was changed every 8 h to remove non-collagenous 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 100 ml l^{-1} aqueous butanol (30 ml g^{-1}) for 24 h with gentle stirring and a change of solution every 8 h. Defatted skins were washed thoroughly with distilled water. The residue was soaked in 0.5 mol kg^{-1} acetic acid (30 ml g^{-1}) for 24 h with gentle stirring. The mixture was then centrifuged at 20 000 × *g* for 1 h. The supernatants were collected and kept at 4 °C. The precipitate was re-extracted in 0.5 mol kg^{-1} acetic acid (30 ml g^{-1}) for 16 h with gentle stirring, followed by centrifugation at 20 000 × *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 mol kg^{-1} in 0.05 mol kg^{-1} Tris–HCl (pH 7.5). The resultant precipitate was collected by centrifugation at 20 000 × *g* for 1 h and then dissolved in 10 vols of 0.5 mol kg^{-1} acetic acid. The solution obtained was dialyzed with 10 vols of 0.1 mol kg^{-1} acetic acid in a dialysis membrane with molecular weight cut-off of 30 kDa for 12 h at 4 °C with a change of solution every 4 h, and subsequently with 10 vols of distilled water with changes of water until neutral pH was obtained. The dialyzate was freeze-dried and referred to as acid-solubilized collagen (ASC). Undissolved residue obtained after acid extraction was thoroughly rinsed with distilled water, suspended (0.5 g ml^{-1}) 0.5 mol kg^{-1} acetic acid and subjected to limited hydrolysis with 100 mg ml^{-1} pepsin (EC 3.4.23.1 powder; 750 U mg^{-1} dry matter, Sigma) for 48 h at 4 °C with gentle stirring. The viscous solution was centrifuged at 20 000 × *g* for 1 h at 4 °C. To terminate the pepsin reaction, the supernatant obtained was dialyzed against 10 vols of 0.02 mol kg^{-1} 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 retentate obtained was centrifuged at 20 000 × *g* for 1 h. The pellet obtained was dissolved (0.1 g ml^{-1}) in 0.5 mol kg^{-1} acetic acid. The solute was further precipitated by addition of NaCl to a final concentration of 2.6 mol kg^{-1} in 0.05 mol kg^{-1} Tris–HCl (pH 7.5). The resultant precipitate was collected by centrifugation at 20 000 × *g* for 1 h and re-dissolved (0.1 g ml^{-1}) in 0.5 mol kg^{-1} acetic acid. The solution was dialyzed and the retentate was freeze-dried in the same manner as with ASC preparation. The dry matter was referred to as pepsin–solubilized collagen (PSC).

Electrophoretic analysis

Protein patterns of collagen samples were analyzed using sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) according to the method of Laemmli.[27] Collagen samples (100 mg) were dissolved in 10 ml 0.02 mol kg^{-1} sodium phosphate buffer (pH 7.2) containing 10 g l^{-1} SDS and 3.5 mol kg^{-1} urea. The sample mixtures were gently stirred at 4 °C for 12 h to dissolve total proteins. Supernatants were collected after centrifuging at 3000 × *g* for 3 min at 4 °C. Solubilized collagen samples were mixed at a ratio of 1:1 (v/v) with the sample buffer (0.5 mol kg^{-1} Tris–HCl, pH 6.8 containing 40 g l^{-1} SDS, 200 ml l^{-1} glycerol with and without 100 ml l^{-1} β-ME). Samples were loaded on the PAGEL®-Compact precast gel (50 g l^{-1} gel) and subjected to electrophoresis at a constant current of 20 mA using a Compact-PAGE apparatus (Atto Co, Tokyo, Japan). After electrophoresis, the gel was stained with 0.5 mg ml^{-1} Coomassie blue R-250 in 150 ml l^{-1} aqueous methanol and 50 ml l^{-1} acetic acid, and destained with 300 ml l^{-1} methanol and 100 ml l^{-1} acetic acid. High molecular weight markers (Sigma) were used to estimate the molecular weights of proteins. Calf skin acid-soluble type I collagen (Sigma), 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 mol kg^{-1} methanesulfonic acid containing 2 ml l^{-1} 3-2(2-aminoethyl)indole at 115 °C for 24 h, and the hydrolyzates were neutralized with 3.5 mol kg^{-1} NaOH and diluted with 0.2 mol kg^{-1} 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*[26] with a slight modification. Freeze-dried samples (0.2 mg) were dissolved in 0.1 ml of 0.1 mol kg^{-1} sodium phosphate, pH 7.2 containing 5 mg ml^{-1} 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^{-1} protein; Wako) 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 75 g l^{-1} 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 solubilities of collagens were determined in 0.5 mol kg^{-1} acetic acid at various pHs and NaCl concentrations according to the method of Montero *et al*[22] with a slight modification. Collagen samples were dissolved in 0.5 mol kg^{-1} acetic acid with gentle stirring at 4 °C for 12 h to obtain final concentrations of 3 and 6 mg ml^{-1}.

To determine the effect of salt concentration on collagen solubility, 5 ml aliquots of collagen solutions (6 mg ml^{-1}) in 0.5 mol kg^{-1} acetic acid were mixed with 5 ml of cold NaCl in acetic acid with various concentrations of 0, 20, 40, 60, 80, 100 and 120 g kg^{-1} to obtain the final NaCl concentrations of 10, 20, 30, 40, 50 and 60 g kg^{-1}, respectively. The mixtures were stirred gently at 4 °C for 30 min and centrifuged at 10 000 × *g* for 30 min at 4 °C. Protein contents in the supernatants were determined by the method of Lowry *et al*[28] using bovine serum albumin as a protein standard. Relative solubilities of collagen samples were 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 solution (3 mg ml^{-1}) was transferred to a centrifuge tube and the pH was adjusted with either 6 mol kg^{-1} NaOH or 6 mol kg^{-1} HCl to obtain final pHs ranging from 1 to 10. The volume of sample solution 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 × *g* for 30 min at 4 °C. Protein contents in the supernatants were determined by the method of Lowry *et al*[28] using bovine serum albumin as a protein standard. Relative solubilities of collagen samples were calculated as previously described.

Thermal transition measurement

Collagen samples were prepared by the method described by Rochdi *et al*[29] with a slight modification. The freeze-dried collagen samples were rehydrated in deionized water or 0.05 mol kg^{-1} acetic acid solution with a sample: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 a 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 20–50 °C with a heating rate of 1 °C min^{-1}. Ice water was used as a cooling medium and the system was equilibrated at 20 °C for 5 min prior to the scan. An empty aluminum pan was used as reference. The maximum transition temperature (T_{max}) was estimated from the maximum peak of the 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.[30] The analysis was performed using a SPSS package (SPSS 8.0 for Windows, SPSS Inc, Chicago, IL, USA).

RESULTS AND DISCUSSION

Isolation of ASC and PSC from bigeye snapper skin

Collagens from bigeye snapper skin were classified as ASC and PSC with yields of 64 g kg^{-1} and 11 g kg^{-1} (wet weight basis), respectively. Collagen in skin was solubilized to a large extent by 0.5 mol kg^{-1} acetic acid extraction. Further solubilization of the remaining residues was achieved by limited pepsin digestion. The result suggested that covalent cross-linking at the telopeptide region of collagen molecules through the condensation of aldehyde groups, as well as the intermolecular cross-linkage of molecules, was not disrupted by acid extraction. These cross-linkages generally caused a decrease in solubility of collagen.[1,31] With the limited pepsin digestion, the cross-linkages at the telopeptide region were cleaved without damaging the integrity of the triple helix. Nevertheless, solubilized collagen with predominantly monomeric molecules could be obtained.[32] It was

found that the major fraction of collagen from bigeye snapper skin was ASC ($850\,g\,kg^{-1}$, based on extractable collagen weight) and a lower content of PSC was found ($150\,g\,kg^{-1}$), suggesting that high inter-molecular cross-linked collagen molecules were present at a low content in the skin of bigeye snapper. The high content of ASC fraction was in accordance with those reported in hake skin (*Merluccius hubbsi*) ($850\,g\,kg^{-1}$ based on extractable collagen)[21] and trout skin (*Salmo irideus* Gibb) ($950\,g\,kg^{-1}$ based on extractable collagen).[22]

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 Fig 1; generally, no differences were observed, indicating that both collagen fractions contained no disulfide bond Mizuta *et al*[8] reported that protein patterns of squid collagen were not changed in the 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. 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 the 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 for hake skin collagen,[21,33] trout,[33] Japanese sea-bass and bullhead shark.[3] Type I collagen consists of two α_1- and one α_2-chain.[34] 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.[2,3,21] 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[1] and starving fish contain collagen with a greater degree of cross-linking than fish that are well fed.[35,36] However, the rate of cross-linking of collagen in fish skins is extremely slow[37] and highly cross-linked molecules are not generally found.[1] The results show that the major collagens in ASC and PSC extracted from bigeye snapper skin were heterotrimers $(\alpha 1)_2\alpha 2$, which contained no disulfide bond.

Amino acid composition

The amino acid compositions of ASC and PSC are shown in Table 1. Glycine was the dominant amino acid in ASC and PSC, accounting for about 237 and $272\,g\,kg^{-1}$ of total amino acids, respectively. Both collagen fractions also contained a high content of hydroxyproline (87 and 80 residues per 1000 residues), hydroxylysine (12 and 13 residues per 1000 residues), and proline (124 and 107 residues per 1000 residues). ASC and PSC showed similar amino acid compositions. Generally, glycine distributes uniformly at every third position throughout most collagen molecules.[1,31,34] The numbers of imino acids, proline and hydroxyproline, in ASC and PSC were 211 and 187 residues per 1000 residues, respectively. The imino acid content in bigeye snapper skin ASC was similar to that observed in calf skin collagen (215 residues per 1000 residues).[38] These amino acids were found to contribute to the stability of collagen fibers

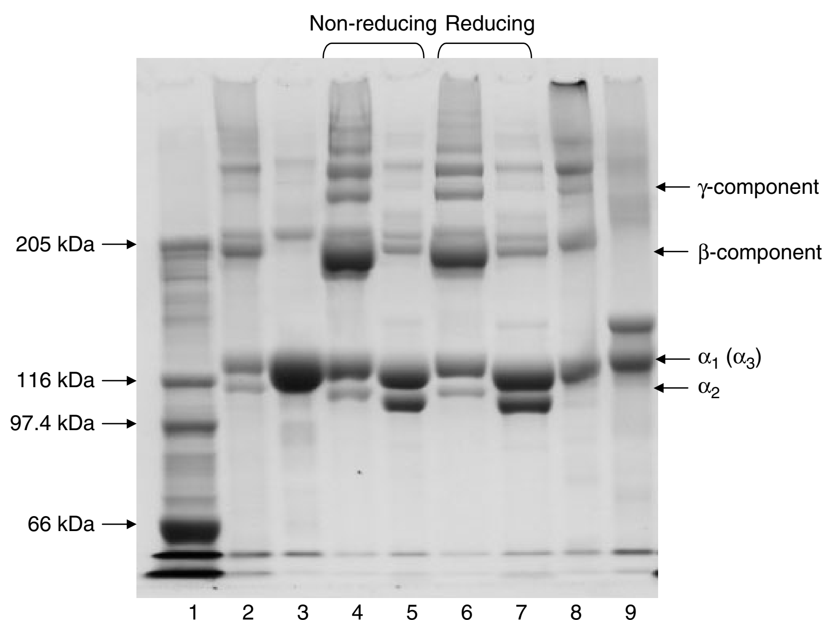

Figure 1. Protein patterns of ASC and PSC from bigeye snapper skin under reducing and non-reducing conditions. Lane 1: high molecular-weight protein markers; lanes 2, 3, 8 and 9, collagen type I, II, III and V, respectively; lanes 4 and 5, ASC and PSC under non-reducing conditions; lanes 6 and 7, ASC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

Table 1. 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

and denaturation temperature.[5] The occurrence of hydroxyproline and hydroxylysine along the collagen molecule was due to the oxidation of hydroxylated residues of proline and lysine, which was catalyzed by proline hydroxylase to form hydroxyproline and hydroxylysine, respectively.[1,34] 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 1000 residues.[2,9,24,33] As shown in this study and previous reports from other species, hydroxyproline content can vary with species and their habitat.[1,35] This might contribute to the differences in properties of collagens among different species.

Peptide mapping of collagen

Peptide maps of ASC and PSC digested by lysyl endopeptidase and V8 protease are shown in Fig 2. 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 limited digestion by V8 protease (lanes 5–7) and lysyl endopeptidase (lanes 8–10) with a concomitant increase in low-molecular-weight peptide fragments. With the digestion by V8 protease, the band intensity of the β-component and higher cross-linked molecules of calf skin collagen type I was slightly decreased and a major fragment with MW 142 kDa was observed. For ASC and PSC, high-molecular-weight cross-links were markedly degraded after digestion with V8 protease. The result suggested that high-molecular-weight 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 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-molecular-weight peptide fragments with MW 101 and 38 kDa were found with calf skin collagen type I, while peptide fragments with MW 96, 84, 61, 38 and 34.6 kDa were generated. Additional peptide fragments with MW 73 kDa for ASC and MW 71 and 58 kDa for PSC were observed.

For peptide mapping of collagens digested by lysyl endopeptidase, all proteins were more extensively

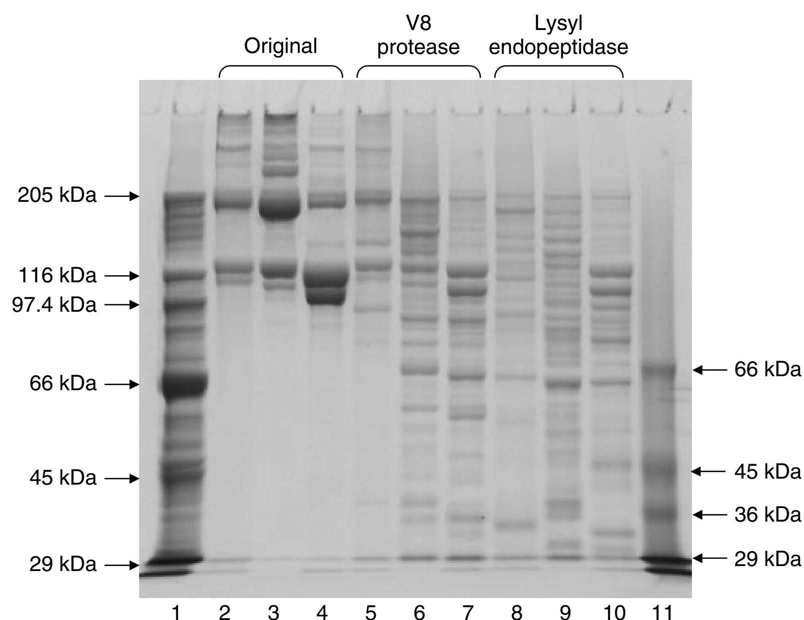

Figure 2. Peptide maps of ASC and PSC from bigeye snapper skin digested by V8 protease and lysyl endopeptidase. Lanes 1 and 11, high- and low-molecular-weight protein markers, respectively; lanes 2, 3 and 4, collagen type I, ASC and PSC; lanes 5, 6 and 7, peptide fragments of collagen type I, ASC and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

hydrolyzed, compared with hydrolysis with V8 protease, as evidenced by the lower original band intensity of each component remaining, with a concomitant increase in lower-molecular-weight peptide fragments. The β-component (175 kDa) and the more highly 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 164, 146 and 139 kDa were generated. A peptide with MW 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. Smaller peptide fragments with MW 105, 97 and 91 kDa were produced in calf skin collagen, ASC and PSC. Although similar molecular-weight peptide fragments were generated among all collagens, some differences in peptide fragments were also found. Fragments with MW 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 87, 76, 68, 54, 46 and 30 kDa were generated in both ASC and PSC. However, only peptide fragments with MW 48, 38, 36 and 35 kDa were observed in ASC, whereas that with MW 32.0 kDa was only found in PSC. Nagai *et al*[9] reported that the peptide map 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).[9,39,40] Peptide mapping was reported to differ among sources and species.[9,41] 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.

Effect of salt concentration on collagen solubility

The effect of NaCl on collagen solubility is shown in Fig 3. Solubility of both ASC and PSC in $0.5\,\text{mol kg}^{-1}$ acetic acid decreased gradually with increasing NaCl concentration up to $30\,\text{g l}^{-1}$ ($p < 0.05$). A sharp decrease in solubility was observed with NaCl concentration above $30\,\text{g l}^{-1}$ ($p < 0.05$). A slight decrease in solubility was also observed with further increase in NaCl concentration (between 50 and $60\,\text{g l}^{-1}$). Montero *et al*[22,42] 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 due to the salting-out effect. When ionic strength increased, the decrease in collagen solubility was possibly caused by an enhanced hydrophobic–hydrophobic interaction, and the competition of ionic salts for water, leading to induced protein precipitation.[43,44] PSC showed slightly greater solubility than ASC at the same NaCl concentration. The greater solubility of PSC might be due to the proteolytic action of pepsin. Additionally, some differences in composition and conformation between the two collagen fractions might result in such a difference. However, both collagen fractions were still soluble in presence of NaCl up to $30\,\text{g l}^{-1}$.

Effect of pH on collagen solubility

The effect of pH on the solubility of ASC and PSC in $0.5\,\text{mol kg}^{-1}$ acetic acid is shown in Fig 4. The highest solubility of ASC and PSC was observed at pH 4 and 5, respectively ($p < 0.05$). In general, ASC and PSC are solubilized to a greater extent at acidic pHs. Drastic decreases in solubility were found for both ASC and PSC fractions with increases in pH up to 6 and 7, respectively. At alkaline pH ranges, solubility increased slightly with increasing pH value up to 10. PSC generally had higher solubility than ASC at all pHs tested except at the pH maximum for ASC, suggesting a lower degree of molecular cross-linking of the PSC fraction, or predominantly weaker

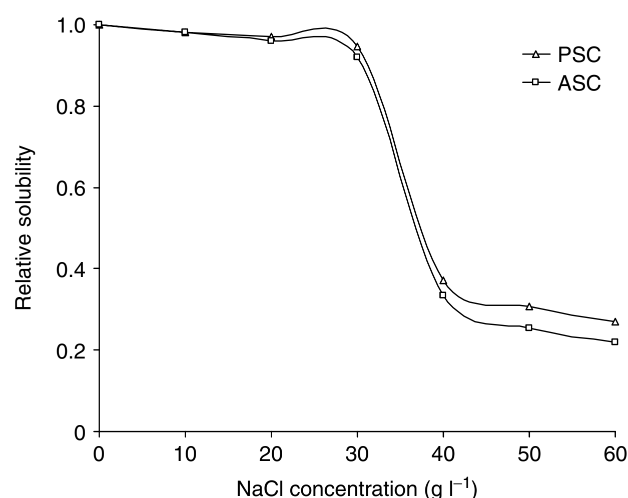

Figure 3. Solubility of ASC and PSC from bigeye snapper skin in $0.5\,\text{mol kg}^{-1}$ acetic acid at different NaCl concentrations.

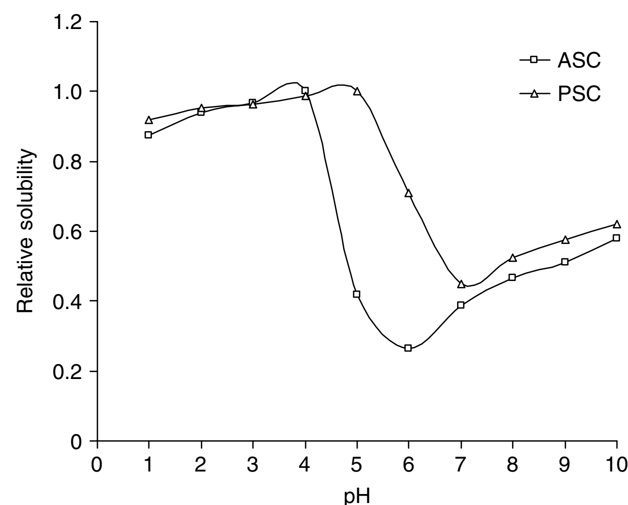

Figure 4. Solubility of ASC and PSC from bigeye snapper skin in $0.5\,\text{mol kg}^{-1}$ acetic acid at different pHs.

bonds than ASC.[33] This result was consistent with the higher contents of cross-linked molecules in ASC shown on SDS–PAGE, compared with that in PSC (Fig 1). The variation in solubility of proteins with pH might be due to differences in isoelectric point. As the pH becomes lower or higher than pI, the repulsion forces between charged residues of protein molecules increase and the solubility of protein is increased.[43] Conversely, hydrophobic-hydrophobic interaction is increased and the total net charge of protein molecules is zero at pI, thereby causing protein precipitation and aggregation.[34,45]

Thermal stability of collagen

Thermal transitions of ASC and PSC in deionized water and 0.05 mol kg^{-1} acetic acid are depicted in Fig 5. Endothermic peaks of ASC and PSC were observed with 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 mol kg^{-1} acetic acid. Similar T_{max} between ASC and PSC in deionized water was observed, suggesting that both fractions had similar denaturation temperatures. Hickman *et al*[32] reported that pepsin cleaved the telopeptide region containing the intermolecular cross-linkages without damaging the integrity of the triple helix. As a consequence, the triple helix structure was still predominant in both ASC and PSC, resulting in similar thermal characteristics for both fractions. However, the denaturation temperature of ASC and PSC from bigeye snapper was much lower than that of mammalian collagen (41 °C).[46,47] The relationship between hydroxyproline content and denaturation temperature has been established. Collagen containing a less hydroxyproline denatured at a lower temperature than that with a higher content. Moreover, hydroxyproline plays an important role in the stabilization of the collagen triple-helix structure due to its hydrogen bonding ability through its -OH group.[1,46] In the presence of 0.05 mol kg^{-1} acetic acid, the peak was shifted to a lower temperature. Thus, acetic acid might change the conformation of collagen and render it more prone to denaturation. The inter-chain hydrogen bonds stabilizing the collagen triple-helix structure were partially cleaved by acetic acid.[48] The denaturation temperature of skin collagen from bigeye snapper was higher than those previously reported for some cold-water fish species. Thermal transition temperatures of skin collagen have been reported for hake (10 °C),[21] Alaska pollack (16.8 °C),[49] Japanese sea bass (26.5 °C), chub mackerel (25.6 °C), bullhead shark (25.0 °C),[3] ocellate puffer (28 °C)[9] and Baltic cod (15 °C).[16] The higher denaturation temperature was attributed to the higher imino acid content of land animal collagen than those in tropical and temperate fish.[2,38,50] Furthermore, the environmental and body temperature in the habitat has been reported to correlate with denaturation temperature of collagen.[49,51–54] Thus, collagen from bigeye snapper skin has high thermal stability, which might be associated with differences in the properties of its collagen from those of other species.

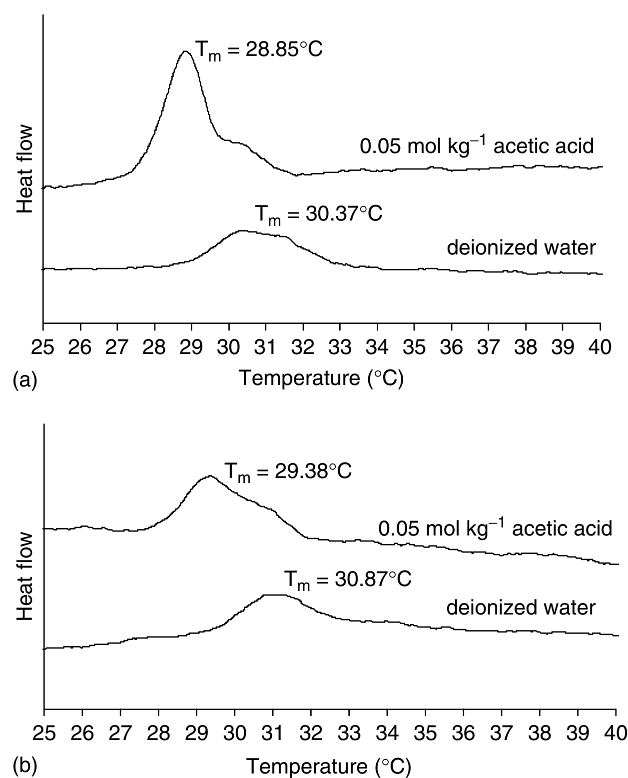

Figure 5. Thermograms of collagen ASC (A) and PSC (B) from bigeye snapper skin rehydrated in 0.05 mol kg^{-1} acetic acid and deionized water.

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