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Isolation and Characterisation of collagen from the skin of brownbanded bamboo shark (*Chiloscyllium punctatum*)

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ABSTRACT

Acid soluble collagen (ASC) and pepsin soluble collagen (PSC) from the skin of brownbanded bamboo shark (*Chiloscyllium punctatum*) were isolated and characterised. The yield of ASC and PSC were 9.38% and 8.86% (wet weight basis), respectively. Based on protein patterns and TOYOPEARL® CM-650M column chromatography, both collagens contained α- and β-chains as their major components. These were characterised as type I collagen with the cross-link of α2-chain. As digested by V8-protease and lysyl endopeptidase, peptide maps of both ASC and PSC were similar, but differed from that of type I collagen from calf skin. Fourier transform infrared (FTIR) spectra of both collagens were similar and pepsin hydrolysis had no effect on triple-helical structure of collagen. Transition temperature (T_{max}) of ASC and PSC were 34.45 and 34.52 °C, respectively, as determined by differential scanning colorimetry (DSC). From zeta potential study, the isoelectric points of ASC and PSC were estimated to be 6.21 and 6.56, respectively. Therefore, the skin of brownbanded bamboo shark could serve as an alternative source of collagen for different applications.

1. Introduction

Shark has been used as food for human consumption and can be further processed into many products. Different parts of sharks can be utilised such as shark meat (fillets and fish ball products), shark fin (dried shark fin), shark liver (shark liver oil contains a large amount of squalene, vitamins A and long-chain omega-3 polyunsaturated fatty acids, shark skin (sun dried products, leather), shark cartilage (shark cartilage powder and shark cartilage chondroitin for food and drug use) (Musick, 2005). Due to their thickness, shark skin can be used as an excellent source of collagen with the unique characteristics.

Collagen is the major fraction of connective tissue and has been used in food, pharmaceutical and photographic industries (Regenstein & Zhou, 2007). Commonly, the main sources for collagen production are pig skin, cattle skin and bone. The outbreak of bovine spongiform encephalopathy (BSE) has resulted in justified anxiety amongst users of cattle collagen. Additionally, the collagen obtained from pig skin and bones cannot be used freely due to the religious constraint (Kittiphattanabawon, Benjakul, Visessanguan, Nagai, & Tanaka, 2005). As a consequence, much attention has been paid to alternative sources of collagen, especially from fish skin and fish bone from the seafood processing industries. So far, skin collagen from several fish species have been isolated and characterised such as bigeye snapper skin (Jongjareonrak, Benjakul, Visessanguan, & Tanaka, 2005), Nile perch skin (Muyonga, Cole, & Duodu, 2004), Baltic cod skin (Sadowska, Kolodziejska, & Niecikowska, 2003) and deep-sea redfish skin (Wang, An, Xin, Zhao, & Hu, 2007). Shark is another elasmobranch, which has been used, for shark fin and fillet production. This processing leads to the generation of skin and cartilage, amongst others, which can be used as a potential raw material for collagen extraction. However, little information is available regarding the collagen from the skin of shark, especially those in the tropical regions. Therefore, the objective of this investigation was to isolate and characterise the collagen from the skin of brownbanded bamboo shark, a by-product from the shark processing plants.

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2. Materials and methods

2.1. Chemicals/enzymes

All chemicals were of analytical grade. Sodium dodecyl sulphate (SDS), Coomassie Blue R-250 and *N*,*N*,*N*′,*N*′-tetramethylethylenediamine (TEMED) were procured from Bio-Rad Laboratories (Hercules, CA, USA). Type I collagen from calf skin, pepsin from porcine stomach mucosa (EC 3.4.23.1), (750 units/mg dry matter), V8-protease from *Staphylococcus aureus* (EC 3.4.21.19) and lysyl endopeptidase from *Achromobacter lyticus* (EC 3.4.21.50) were purchased from Sigma Chemical Co. (St. Louis, MO, USA). Type II collagen from porcine cartilage was obtained from Wako Pure Chemical Industries, Ltd. (Tokyo, Japan). High-molecular-weight markers and TOYOPEARL® CM-650M were purchased from GE Healthcare UK Limited (Buckinghamshire, UK) and Tosoh Corporation (Tokyo, Japan), respectively.

2.2. Shark skin preparation

Skin of brownbanded bamboo shark (*Chiloscyllium punctatum*) with the size of 70–100 cm in length was obtained from Blue Ocean Food Products Co., Ltd. in Samutsakhon Province of Thailand. The frozen shark skin (10 kg) packed in polyethylene bags (1 kg/bag) was placed in ice at a ratio of skin to ice of 1:2 (w/w) using a polystyrene box as a container. The skin was transported to the Department of Food Technology, Prince of Songkla University by bus over a 10 h period. Upon arrival, the skin was kept at −20 °C until use, usually within one week. Two different lots of shark skin were used. For each lot, the extraction was carried out in duplicate.

To prepare collagen from shark skin, the frozen skin was thawed with running water until the core temperature reached 5 °C. Thereafter, it was washed with cold tap water (⩽10 °C). The residual meat on shark skin was removed by knife and washed with cold tap water until any residual smell of ammonia disappeared. The clean shark skin was cut into small pieces (1.0×1.0 cm^2) using a pair of scissors.

2.3. Assay for proteolytic activity of pepsin

Proteolytic activity of porcine pepsin was determined using haemoglobin as a substrate according to the method of Nalinanon, Benjakul, Visessanguan, and Kishimura (2008). To initiate the reaction, 200 μl of porcine pepsin solution were added to the assay mixture containing 200 μl of 2% haemoglobin, 200 μl of distilled water and 625 μl of McIlvaine's buffer (0.2 M Na-phosphate and 0.1 M Na-citrate), pH 2. Appropriate dilution was made to ensure that the amount of enzyme was not excessive for the available substrate in the assay system. The reaction was conducted at pH 2.0 and 50 °C for 20 min. To terminate enzymatic reaction, 200 μL of 50% (w/v) trichloroacetic acid (TCA) were added. Unhydrolysed protein substrate was allowed to precipitate for 15 min at 4 °C, followed by centrifugation at 4725*g* for 10 min using a MIKRO 20 centrifuge (Hettich Zentrifugen, Tuttlingen, Germany). The oligopeptide content in the supernatant was determined by the method of Lowry (Lowry, Rosebrough, Farr, & Randall, 1951) using tyrosine as a standard. One unit of activity was defined as that releasing 1 μmole of tyrosine per min (μmol Tyr/min). A blank was run in the same manner, except that pepsin was added into the reaction mixture after the addition of 50% TCA (w/v).

2.4. Extraction of collagen from shark skin

Acid soluble collagen (ASC) and pepsin soluble collagen (PSC) were extracted from the prepared shark skin following the method of Kittiphattanabawon et al. (2005) and Nalinanon, Benjakul, Visessanguan, and Kishimura (2007) with a slight modification. All procedures were carried out at 4 °C.

2.4.1. Pretreatment of shark skin

To remove non-collagenous proteins, the prepared shark skin was mixed with 0.1 M NaOH at a solid to alkali solution ratio of 1:10 (w/v). The mixture was continuously stirred for 6 h. The alkali solution was changed every 2 h. Then, the deproteinised skin was washed with cold water until a neutral or faintly basic pH of wash water was reached.

2.4.2. Extraction of acid soluble collagen

Pretreated skin was soaked in 0.5 M acetic acid with a solid to solvent ratio of 1:15 (w/v) for 48 h with continuous stirring using an overhead stirrer model W20.n (IKA®-Werke GmbH & CO.KG, Stanfen, Germany). The mixtures were filtered with two layers of cheesecloth. The collagen in the filtrate was precipitated by adding NaCl to a final concentration of 2.6 M in the presence of 0.05 M tris(hydroxymethyl) aminomethane at pH 7.5. The resultant precipitate was collected by centrifugation at 20,000*g* at 4 °C for 60 min using a refrigerated centrifuge model Avanti® J-E (Beckman Coulter, Inc., Palo Alto, CA, USA). The pellet was dissolved in a minimum volume of 0.5 M acetic acid, dialysed against 25 volumes of 0.1 M acetic acid for 12 h. Thereafter, it was dialysed against 25 volumes of distilled water for 48 h. The resulting dialysate was freeze dried and referred to as "acid soluble collagen, ASC".

2.4.3. Extraction of pepsin soluble collagen

Non-dissolved residue obtained after acid extraction was used for pepsin soluble collagen extraction. The residue was soaked in 0.5 M acetic acid with a solid to solvent ratio of 1:15 (w/v) and porcine pepsin (20 unit/g of residue) was added. The mixtures were continuously stirred at 4 °C for 48 h, followed by filtration with two layers of cheesecloth. The filtrate was collected and subjected to precipitation and dialysis in the same manner with that used for ASC as previously described. The obtained collagen was referred to as "pepsin soluble collagen, PSC".

2.5. Proximate analysis

Shark skin, ASC and PSC were subjected to proximate analyses, including moisture, ash, fat and protein contents, according to the AOAC (2000) method Nos. 950.46, 928.08, 960.39 and 920.153, respectively.

2.6. UV–Vis measurement

Collagen was dissolved in 0.5 M acetic acid to obtain a concentration of 1 mg/ml. The solution was then subjected to UV–Vis measurement using a double-beam spectrophotometer (UV-1700, Shimadzu, Kyoto, Japan). Prior to measurement, the base line was set with 0.5 M acetic acid. The spectrum was obtained by scanning the wavelength in the range of 220–600 nm with a scan speed of 50 nm/min at room temperature.

2.7. Amino acid analysis

ASC and PSC were hydrolysed under reduced pressure in 4.0 M methane sulphonic acid containing 0.2% (v/v) 3–2(2-aminoethyl)indole at 115 °C for 24 h. 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).

2.8. SDS–polyacrylamide gel electrophoresis (SDS–PAGE)

SDS–PAGE was performed by the method of Laemmli (1970). The samples were dissolved in 5% SDS solution. The mixtures were then heated at 85 °C for 1 h using a temperature controlled water bath model W350 (Memmert, Schwabach, Germany), followed by centrifugation at 8500*g* for 5 min using a microcentrifuge (MIKRO20, Hettich Zentrifugan, Germany) to remove undissolved debris. Solubilised samples were mixed at 1:1 (v/v) ratio with sample buffer (0.5 M tris–HCl, pH 6.8 containing 4% SDS and 20% glycerol in the presence or absence of 10% (v/v) βME). Samples were loaded onto a polyacrylamide gel made of 7.5% separating gel and 4% stacking gel and subjected to electrophoresis at a constant current of 20 mA/gel. After electrophoresis, gels were fixed with a mixture of 50% (v/v) methanol and 10% (v/v) acetic acid for 30 min, followed by staining with 0.05% (w/v) Coomassie Blue R-250 in 15% (v/v) methanol and 5% (v/v) acetic acid for 1 h. Finally, they were destained with the mixture of 30% (v/v) methanol and 10% (v/v) acetic acid for 1 h and destained again with the same solution for 30 min. High-molecular-weight protein markers (GE Healthcare UK Limited, Buckinghamshire, UK) were used to estimate the molecular weight of proteins. Type I and type II collagens from calf skin and porcine cartilage, respectively, were used as standard collagens.

2.9. TOYOPEARL® CM-650M column chromatography

TOYOPEARL® CM-650M column chromatography was carried out according to the method of Nagai, Suzuki, and Nagashima (2008) with a slight modification. Collagen samples (20 mg) were dissolved in 5 ml of starting buffer (20 mM sodium acetate buffer, pH 4.8) and boiled for 1 min. The mixtures were centrifuged at 20,000*g* at room temperature (25–26 °C) for 30 min. The supernatants were applied to a TOYOPEARL® CM-650M column (1.0 × 5.0 cm) previously equilibrated with 10 volumes of the starting buffer at a flow rate of 60 ml/h. After loading, the unbound proteins were washed by the same buffer until A_{230} was less than 0.05. Elution was achieved with a linear gradient of 0–0.3 M NaCl in the same buffer at a flow rate of 40 ml/h with a total volume of 200 ml. The eluant was monitored at 230 nm and 2 ml fractions were collected. The selected fractions were subjected to SDS–PAGE using 7.5% separating gel and 4% stacking gel as previously described.

2.10. Peptide mapping of collagen from shark skin

Peptide mappings of collagen samples were performed according to the method of Kittiphattanabawon et al. (2005) with a slight modification. The samples (6 mg) were dissolved in 1 ml of 0.1 M sodium phosphate, pH 7.2 containing 0.5% (w/v) SDS. Then, the mixtures were preheated at 37 °C for 3 h and 300 μl of them were transferred to test tubes for digestion. To initiate the digestion, 20 μl of each enzyme solution, including *S. aureus* V8-protease or lysyl endopeptidase from *A. lyticus*, with concentrations of 5 and 50 μl/ml, respectively, were added to the mixtures. The reaction mixtures were then incubated at 37 °C for 1 h. The reactions were 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% separating gel and 4% stacking gel. Peptide mapping of calf skin collagen acid-soluble type I was also conducted in the same manner and the peptide patterns were compared.

2.11. Fourier transform infrared (FTIR) spectroscopy

FTIR spectra of collagens were obtained using a Bruker model EQUINOX 55 FTIR spectrometer (Bruker, Ettlingen, Germany) equipped with a deuterated l-alanine tri-glycine sulphate (DLATGS) detector. The Horizontal Attenuated Total Reflectance Accessory (HATR) was mounted into the sample compartment. The internal reflection crystal (Pike Technologies, Madison, WI, USA), made of zinc selenide, had a 45° angle of incidence to the IR beam. Spectra were acquired at a resolution of 4 cm^{-1} and the measurement range was 4000–650 cm^{-1} (mid-IR region) at room temperature. Automatic signals were collected in 32 scans at a resolution of 4 cm^{-1} and were rationed against a background spectrum recorded from the clean, empty cell at 25 °C. Analysis of spectral data was carried out using a OPUS 3.0 data collection software programme (Bruker, Ettlingen, Germany).

2.12. Differential scanning calorimetry (DSC)

The collagens were rehydrated by adding deionised distilled water to dried samples at a solid to solution ratio of 1:40 (w/v). The mixtures were allowed to stand for 2 days at 4 °C prior to analysis. Differential scanning calorimetry (DSC) was performed using a differential scanning calorimeter model DSC 7 (Perkin Elmer, Norwalk, CT, USA). Calibration was run using Indium thermogram. The sample (5–10 mg) was accurately weighed into aluminium pans and sealed. The sample was scanned at 1 °C/min over the range of 20–50 °C using iced water as the cooling medium. An empty pan was used as the reference. The maximum transition temperature (T_{max}) was estimated from the thermogram. Total denaturation enthalpy (ΔH) was estimated by measuring the area of DSC thermogram.

2.13. Zeta potential analysis

Collagens were dissolved in 0.5 M acetic acid to obtain a final concentration of 0.05% (w/v). The mixtures were continuously stirred at 4 °C using a magnetic stirrer model BIG SQUID (IKA®-Werke GmbH & CO.KG, Stanfen, Germany) until the samples were completely solubilised.

Zeta (ζ) potential of collagen solutions was measured by Zeta potential analyser model ZetaPALs (Brookhaven Instruments Co., Holtsville, NY, USA). Solutions (20 ml) were transferred to autotitrator model BI-ZTU (Brookhaven Instruments Co., Holtsville, NY, USA), in which the pH of solutions were adjusted to 2–12 using either 1.0 M nitric acid or 1.0 M KOH. The obtained zeta potential of solution at all pHs determined was recorded.

3. Results and discussion

3.1. Proximate analysis of brownbanded bamboo shark skin, ASC and PSC

Shark skin contained 61.96% moisture as its major component, followed by protein (24.75%) and ash (12.12%). Trace amounts of fat (0.19%) were found in the skin. Moisture, protein, fat and ash contents of Nile perch skin were 68.4%, 21.6%, 6.8% and 6.0%, respectively (Muyonga et al., 2004). Thus, the composition of skin varies with fish species and might affect the extraction of collagen differently. When ASC and PSC were extracted from skin, both collagens had a similar composition with low ash and fat contents. Both collagens showed high protein content (89.81–89.89%) with a moisture content of 7.77–8.09%. From UV–Vis spectra of both collagens, an absorbance at 230 with high intensity was observed with no absorption peak at 280 nm (data not shown). The results indicated high efficacy of non-collagenous protein removal. Collagen commonly has a low amount of tyrosine, which could absorb UV-light at 280 nm (Duan, Zhang, Du, Yao, & Konno, 2009).

Yields of 9.38% and 8.86% (wet weight basis) were obtained for ASC and PSC, respectively. The results obtained suggested that collagen in skin was not completely extracted with 0.5 M acetic acid. Cross-link mediated by covalent bonds through the condensation of aldehyde groups at the telopeptide region as well as the inter-molecular cross-links might lead to a decrease in the solubility of collagen in the acidic solution used for extraction (Jongjareonrak, Benjakul, Visessanguan, Nagai, & Tanaka, 2005). The residues after acetic acid extraction were further solubilised by pepsin digestion, in which the telopeptide region was cleaved and inter-molecular cross-links could be hydrolysed (Balian & Bowes, 1977). The extractable yield (sum of yield of ASC and PSC) of shark skin collagens (18.24% wet weight) was much higher than that of brownstripe red snapper (13.7% wet weight) and bigeye snapper (7.5% wet weight) (Jongjareonrak, Benjakul, Visessanguan, Nagai, et al., 2005; Jongjareonrak, Benjakul, Visessanguan, & Tanaka, 2005). The results suggested that shark skin might have a loosened matrix, via swelling mechanism in acidic solution, leading to the ease of pepsin to cleave the telopeptide region. As a consequence, a higher yield was obtained.

3.2. Amino acid composition of collagens from the skin of brownbanded bamboo shark

ASC and PSC showed similar amino acid compositions (Table 1). Both collagens had glycine (318–323 residues/1000 residues) as their major amino acid, followed by proline (111–113 residues/1000 residues), alanine (105–106 residues/1000 residues) and hydroxyproline (93–94 residues/1000 residues). About one-third of the total amino acid residues of collagen were glycine with about 12% proline, 11% alanine and 10% hydroxyproline (Pearson & Young, 1989). The imino acid content (proline + hydroxyproline) of ASC and PSC was 204 and 207 resiudes/1000 residues, respectively, which was slightly lower than that of calf skin collagen (215 residues/1000 residues), but much higher than that of cod skin collagen (154 residues/1000 residues) (Duan et al., 2009). The difference in imino acid content amongst animals was associated with the difference in the living environments of their sources, particularly habitat temperature (Regenstein & Zhou, 2007). Additionally, the imino acid content was reported to have a major influence on thermal stability of collagen (Muyonga et al., 2004). The stability of collagen was proportional to the total content of pyrrolidine imino acids. Pro + Hyp rich zones of the molecules are most likely involved in the formation of junction zones stabilized by hydrogen bonding (Johnston-Banks, 1990). Hydroxylysine (5–6 residues/1000 residues) was found in both ASC and PSC from shark skin. Hydroxylysine might undergo cross-linking, leading to the compact structure of shark skin (Balian & Bowes, 1977).

Table 1
Amino acid compositions of ASC and PSC from the skin of brownbanded bamboo shark (residues/1000 residues).

Amino acid	ASC	PSC
Alanine	105	106
Arginine	51	51
Aspartic acid/asparagine	42	41
Cysteine	1	1
Glutamine/glutamic acid	77	75
Glycine	318	323
Histidine	7	7
Isoleucine	18	18
Leucine	24	23
Lysine	29	28
Hydroxylysine	6	5
Methionine	12	12
Phenylalanine	14	13
Hydroxyproline	93	94
Proline	111	113
Serine	41	40
Threonine	23	23
Tyrosine	3	2
Valine	25	25
Total	**1000**	**1000**
Imino acid	204	207

3.3. Protein patterns and subunit compositions of collagens from the skin of brownbanded bamboo shark

Protein patterns of ASC and PSC determined under non-reducing and reducing conditions are shown in Fig. 1. The same protein patterns for both collagens analysed under both conditions was observed. It was indicated that both ASC and PSC contained a negligible amount of cysteine, which contributed to disulphide bond formation (Kittiphattanabawon et al., 2005). This result was in accordance with those of collagen from other fish skin (Jongjareonrak, Benjakul, Visessanguan, Nagai, et al., 2005; Jongjareonrak, Benjakul, Visessanguan, & Tanaka, 2005; Kittiphattanabawon et al., 2005; Muyonga et al., 2004; Nalinanon et al., 2007). Both ASC and PSC possessed a similar protein pattern, comprising α1- and β-chains, as their major proteins. Nevertheless, the faint band of α2-chain was noticeable in both ASC and PSC. Furthermore, γ-chain was also found in both collagens in a small amount.

The elution profiles of ASC and PSC on the TOYOPEARL® CM-650M column after being dissociated with heat treatment are shown in Fig. 2. The chromatograms of both ASC and PSC were quite similar. The chromatographic fractions indicated by the numbers were subjected to SDS–PAGE. The fractions of ASC (Fig. 2A) and PSC (Fig. 2B) were eluted as 2 major peaks. The α1-chain was found in the first peak (fraction Nos. 28–40 and 23–36 for ASC and PSC, respectively) whilst the α2- and β-chains were found in the second peak (fraction Nos. 41–59 and 37–55 for ASC and PSC, respectively). The results revealed that both collagens might be type I collagen, although the band intensity of α1-chain was not 2-fold higher than that of α2-chain (Fig. 1). It was suggested that α2 component might dimerise into the β-component and form β_{12}-dimer (Hwang, Mizuta, Yokoyama, & Yoshinaka, 2007). As a result, much lower band intensity of α2-chain was detected on SDS–PAGE. Similar results have been reported for collagen type I from other elasmobranches (Bae et al., 2008; Hwang et al., 2007).

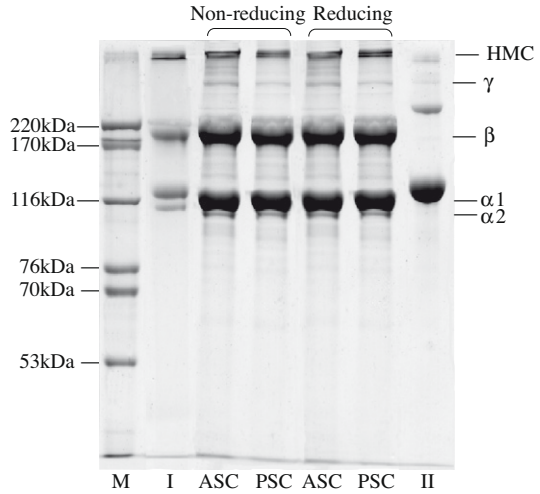

Fig. 1. SDS–PAGE patterns of ASC and PSC from the skin of brownbanded bamboo shark under non-reducing and reducing conditions. M, I, II and HMC denote high-molecular-weight protein markers, type I collagen from calf skin, type II collagen from porcine cartilage and high MW cross-linked components, respectively.

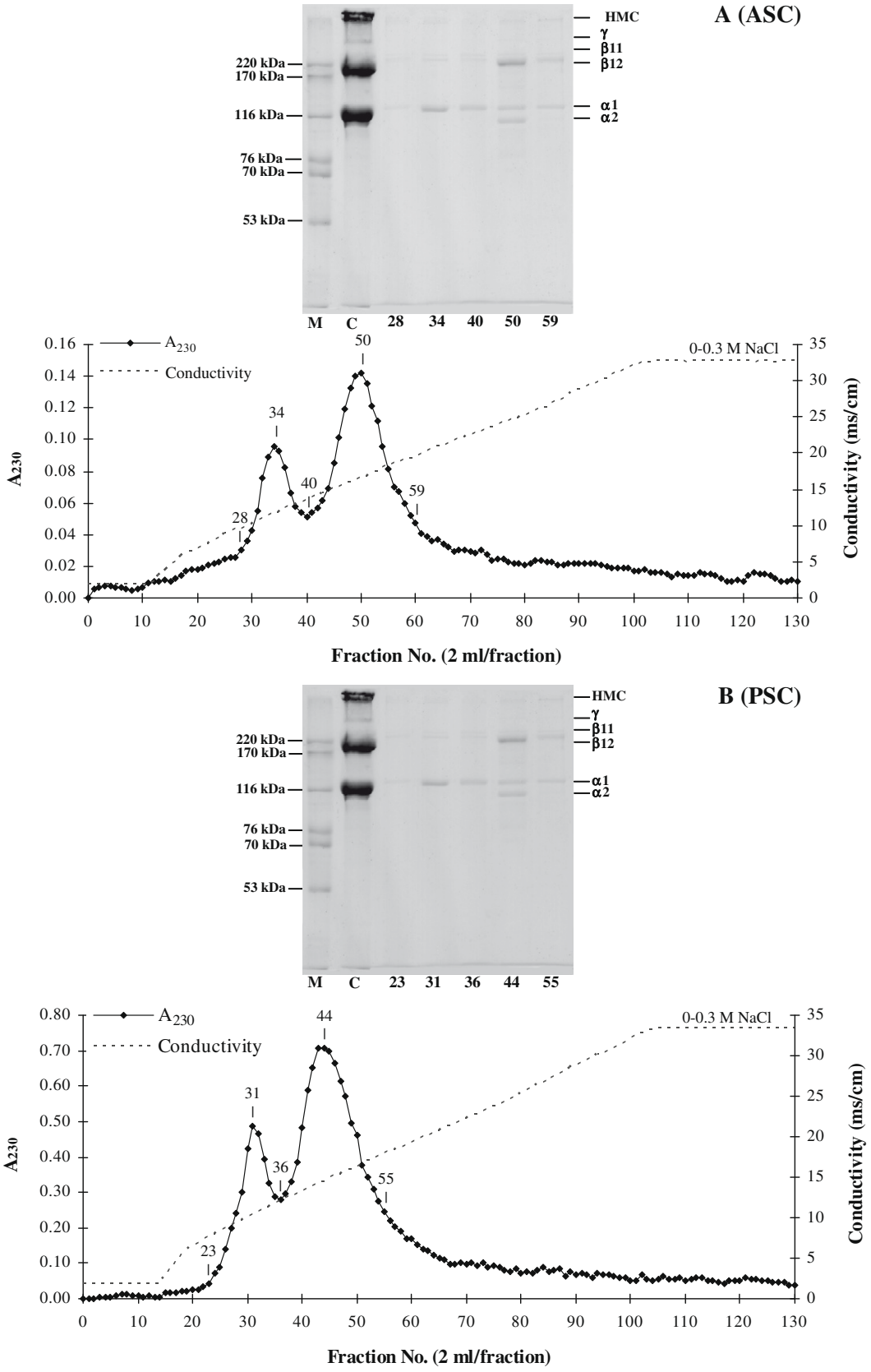

Fig. 2. Elution profile of ASC (A) and PSC (B) from the skin of brownbanded bamboo shark on the TOYOPEARL® CM-650M ion-exchange column. The fractions indicated by numbers were examined by SDS–PAGE using 7.5% separating gel and 4% stacking gel. M, C and HMC denote high-molecular-weight protein markers, collagen and high MW cross-linked components, respectively.

3.4. Peptide mapping of collagen from the skin of brownbanded bamboo shark

The peptide maps of shark skin collagen digested by lysyl endopeptidase and V8-protease in comparison with type I collagen from calf skin are shown in Fig. 3. For peptide maps of collagens digested by lysyl endopeptidase (lane 5–7), all components, including α1-, β-, γ-chains and high MW cross-linked components, were markedly hydrolysed and the degradation peptides with MW of 209.1, 163.4, 153.8, 136.3, 97.6, 90.4, 77.5, 73.3, 64.6, 61.3, 54.4, 50.2 and 47 kDa were obtained. Calf skin collagen was more susceptible to hydrolysis than shark skin collagen as evidenced by the lower band intensity of each component retained. Peptides with MW of 220, 188.6, 170, 160.1, 93.9, 76, 73.3, 69.1, 58.9 and 53 kDa were generated after digestion. Jekel, Weijer, and Beintema (1983) reported that lysyl endopeptidase is a serine endoprotease which hydrolyses peptide bonds at the carboxyl side of lysyl residues. The result suggested that calf skin collagen might contain a higher lysine content than shark skin collagen.

For peptide maps of collagens digested by V8-protease, β-chain and high MW cross-linked components were degraded to some degrees, whereas γ-chain was markedly degraded and α-chain was partially hydrolysed into 11 major peptide fragments with MW ranging from 48.9 to 109.5 kDa. Calf skin collagen was resistant to digestion by V8-protease. V8-protease exhibits a high degree of specificity for glutamic acid and aspartic acid residues of peptides and proteins (Vercaigne-Marko, Kosciarz, Nedjar-Arroume, & Guillochon, 2000). Thus, collagen from calf skin might contain a lower amount of these amino acid residues, compared with ASC and PSC (116–119 residues/1000 residues).

Based on peptide maps of collagens digested by each protease, similar peptide maps between ASC and PSC were observed, whilst a distinct difference was found, compared with that of calf skin collagen. As a result, it was implied that shark skin collagen and calf skin collagen were completely different, mainly in their primary structures, especially amino acid sequence and composition.

3.5. Fourier transform infrared (FTIR) spectra of collagen from the skin of brownbanded bamboo shark

FTIR spectra of ASC and PSC from the shark skin are shown in Fig. 4A. The major peaks in the spectra of both ASC and PSC from the skin of brownbanded bamboo shark were similar to those of collagen from others fish species (Muyonga et al., 2004; Nagai et al., 2008; Wang et al., 2007). Similar FTIR spectra were observed between ASC and PSC. The amide A band of ASC and PSC were found at 3292 and 3294 cm^{-1}, respectively. This band is generally associated with the N–H stretching vibration and shows the existence of hydrogen bonds. Doyle, Blout, and Bendit (1975) reported that a free N–H stretching vibration commonly occurs in the range of 3400–3440 cm^{-1}. When the NH group of a peptide is involved in a hydrogen bond, the position is shifted to lower frequencies. Amide B band of both collagens was observed at 2921–2925 cm^{-1}, in agreement with that reported by Nagai et al. (2008).

The sharp amide I band of ASC and PSC was observed at 1631 and 1635 cm^{-1}, respectively. This band is associated with C=O stretching vibration or hydrogen bond coupled with COO^- (Payne & Veis, 1988). The amide I peak underwent a decrease in absorbance, followed by a broadening accompanied by the appearance of additional shoulders when collagen was heated at higher temperature (Bryan et al., 2007). Due to the similarity in the amplitude, both collagens were most likely not denatured during the extraction. This was reconfirmed by the ratio of approximately 1 between amide III and 1454 cm^{-1} band of both collagens. Ratio of approximately 1 revealed the triple-helical structure of collagen (Plepis, Goissis, & Das-Gupta, 1996). The amide II of both collagens ap-

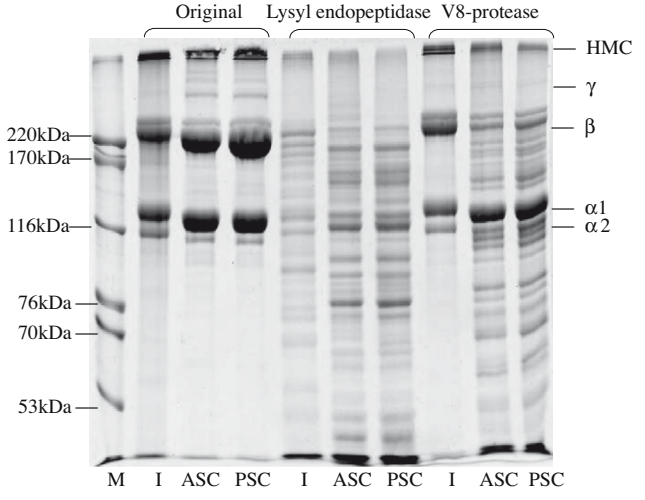

Fig. 3. Peptide maps of ASC and PSC from the skin of brownbanded bamboo shark digested by lysyl endopeptidase or V8-protease. M and I and HMC denote high-molecular-weight protein markers, type I collagen from calf skin and high MW cross-linked components, respectively.

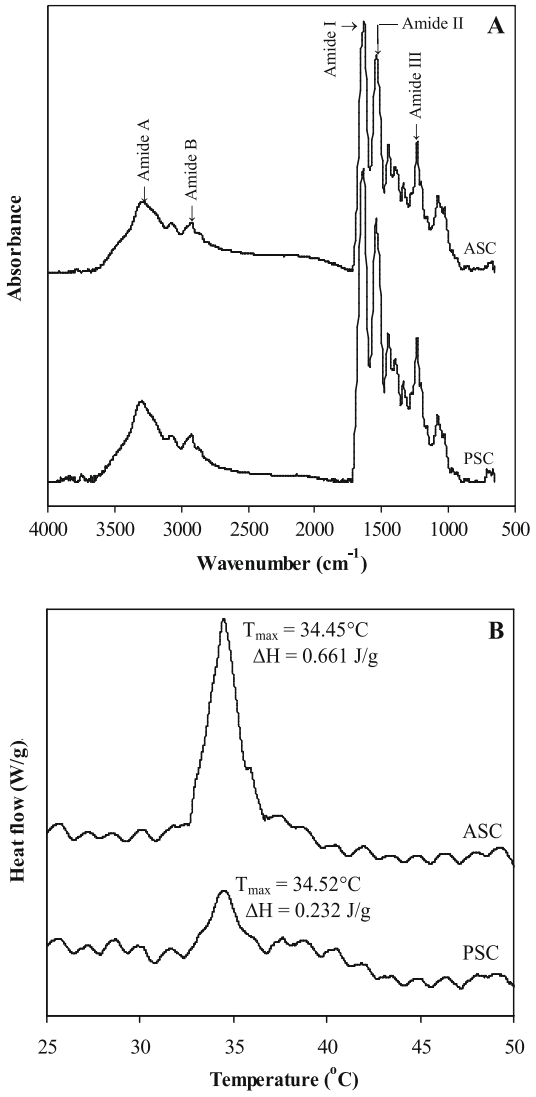

Fig. 4. FTIR spectra (A) and DSC thermogram (B) of ASC and PSC from the skin of brownbanded bamboo shark.

peared at 1538–1541 cm^{-1}, resulting from N–H bending vibration coupled with CN stretching vibration (Krimm & Bandekar, 1986). Thus, both ASC and PSC showed a similar secondary structure.

3.6. Thermal transition of collagen from the skin of brownbanded bamboo shark

The maximum endothermic temperature (T_{max}) of ASC and PSC was 34.45 and 34.52 °C, respectively (Fig. 4B). This is quite similar to that of collagen from other elasmobranches such as eagle ray (34.1 °C), red stingray (33.2 °C) and yantai stingray (32.2 °C) (Bae et al., 2008). Since T_{max} of both ASC and PSC was not different, pepsin digestion might not affect collagen structure, especially triple-helical structure. ASC and PSC from brownstripe red snapper skin showed similar T_{max} (Jongjareonrak, Benjakul, Visessanguan, Nagai, et al., 2005). Nevertheless, T_{max} of ASC and PSC from the skin of brownbanded bamboo shark were lower than that of mammalian collagen such as porcine skin collagen (37 °C) (Nagai et al., 2008). In contrast, it was much higher than that of cold-water fish skin, including cod skin (15 °C) and deep-sea redfish (16.1 °C) (Sadowska et al., 2003; Wang et al., 2007), and that of other tropical fish such as brownstripe red snapper (31.5 °C) and bigeye snapper (30.4 °C) (Jongjareonrak, Benjakul, Visessanguan, Nagai, et al., 2005; Jongjareonrak, Benjakul, Visessanguan, & Tanaka, 2005). The difference in T_{max} amongst collagens from those species was correlated with the imino acid content (proline and hydroxyproline), body temperature and environmental temperature (Kittiphattanabawon et al., 2005; Nagai et al., 2008; Pearson & Young, 1989). ASC and PSC from the skin of brownbanded bamboo shark contained a higher amount of imino acids, compared with those from the skin of cod (154 residues/1000 residues, Duan et al., 2009), deep-sea redfish (160 residues/1000 residues, Wang et al., 2007), bigeye snapper (193 residues/1000 residues, Kittiphattanabawon et al., 2005) and carp (190 residues/1000 residues, Duan et al., 2009). However, it was noted that PSC (0.232 J/g) had a much lower enthalpy (ΔH) than did ASC (0.661 J/g). The cleavage of telopeptide region by pepsin or removal of some of those peptides might facilitate the denaturation of PSC induced by heat.

3.7. Zeta potentials of collagen from the skin of brownbanded bamboo shark

The zeta potentials of ASC and PSC solutions at different pHs are shown in Fig. 5. The zero surface net charge of ASC and PSC was observed at pH of 6.21 and 6.56, respectively. Vojdani (1996) reported that a protein in an aqueous system has a zero net charge at its isoelectric point (pI), when the positive charges are balanced out by the negative charges (Bonner, 2007). Thus, pI of ASC and PSC were estimated to be 6.21 and 6.56, respectively. The difference in pI between ASC and PSC might be associated with the partial removal of telopeptides by the pepsin used.

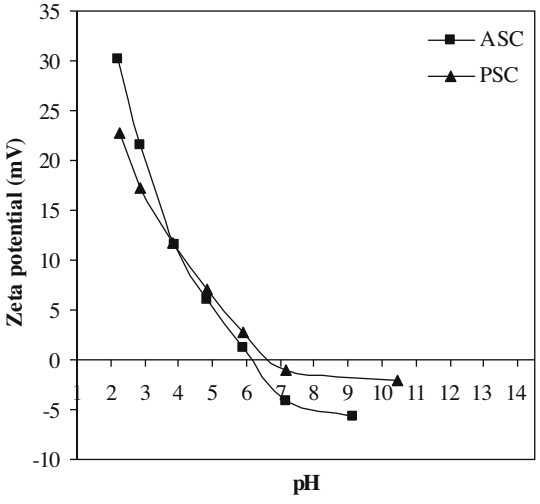

Fig. 5. Zeta (ζ) potential of ASC and PSC from the skin of brownbanded bamboo shark at different pHs.

4. Conclusions

Extraction of collagens from the skin of brownbanded bamboo shark could be achieved by acid solubilisation either in the absence or presence of pepsin. Shark skin collagen was different from that from other fish, in which α2-chain was covalently cross-linked to β-chain. Both ASC and PSC were quite similar in terms of their primary and secondary structures.

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