

## Isolation and characterization of thermostable collagen from the marine eel-fish (*Evenchelys macrura*)



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### ABSTRACT

**Aim and methods:** Collagen is the most abundant protein found in animal body, which is widely used for biomedical and pharmaceutical applications. In the present study, acid soluble collagen (ASC) and pepsin soluble collagen (PSC) from the skin wastes of marine eel fish (*Evenchelys macrura*) were isolated and characterized.

**Results:** ASC and PSC extracted from eel fish skin showed the yields of 80 and 7.10 percent (based on dry weight), respectively. ASC and PSC comprising different  $\alpha$ -chains ( $\alpha_1$ ,  $\alpha_2$  and  $\alpha_3$ ) were characterized as type I and exhibited high solubility in acidic pH (1–4) and were soluble in the presence of NaCl at concentration up to 3.0 and 4.0 percent (w/v) for ASC and PSC, respectively. Amino acids analysis of both ASC and PSC contained imino acid of 190 and 200 residues per 1000 residues, respectively. The present results of ASC and PSC from eel fish skin exhibited higher thermal stability of 39 °C and 35 °C, respectively. Similar, Fourier transform infrared (FTIR) spectra of ASC and PSC were observed and suggesting that pepsin hydrolysis did not affect the secondary structure of collagen, especially triple-helical structure.

**Conclusion:** These results suggest that the marine eel fish skin collagen close to the  $T_d$  (denaturation temperature) of mammalian collagen which could be used in the biomedical materials, food and pharmaceutical industries as an alternative source.

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### 1. Introduction

A greater amount of food has been dumped as commercial and domestic waste. Although there is an attempt to decrease the waste in the world, the quantity of the waste produced is increasing annually [1]. Recently, there has been much interest in investigating possible means of making more effective use of under-utilized resources and industrial wastes [1]. Marine captured fisheries contribute 50 percent of total world fish production and more than 70 percent were utilized by processing industries. The annual discarding rate of world fisheries were estimated at 25 percent of the total marine captured fisheries [2].

Collagen is unique in its ability to form insoluble fibers that have a high tensile strength and right-handed triple superhelical rod consisting of three polypeptide chains and is found in connective tissues, including tendons, bones and skins (e.g. type I collagen) [3]. Collagen has been, traditionally, isolated from the skins of land-based animals, such as cow and pig. Non-denatured collagens from these sources find applications in food, cosmetics, biomedical, and pharmaceutical industries. Denatured collagen, known as gelatin, finds applications in the food and biomedical industries. Biomedical and pharmaceutical applications of collagen include the treatment of hypertension, urinary incontinence and pain associated with osteoarthritis, use in tissue engineering for implants in humans, inhibition of angiogenic diseases, such as diabetes complications, obesity, and arthritis. In recent years, the outbreak of bovine spongiform encephalopathy (BSE) transmissible spongiform encephalopathy (TSE), and the foot-and-mouth disease (FMD) crisis, the uses of collagen and collagen derived products of land animal origin have become of more concern. In addition, the collagens extracted from bovine sources are prohibited for Sikhs and Hindus, whilst porcine collagen cannot be consumed by Muslims and Jews, both of whom require bovine to be religiously prepared [4,5].

As a consequence, the alternative sources of collagen, especially from aquatic animals including freshwater and marine fish and mollusks have received increasing attention. Furthermore the use

**Abbreviations:** ASC, acid-soluble collagen; PSC, pepsin-solubilized collagen; NaCl, sodium chloride; NaOH, sodium hydroxide; Tris-HCl, tris hydrochloride; BSA, bovine serum albumin;  $\beta$ ME,  $\beta$ -mercaptoethanol; SDS-PAGE, sodium-dodecyl sulfate-polyacrylamide gel electrophoresis; DEAE, diethylaminoethyl cellulose-52; HPLC, high performance liquid chromatography (or high pressure liquid chromatography); KBr, potassium bromide; kDa, kiloDalton;  $T_d$ , denaturation temperatures; FTIR, Fourier transform infrared.

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of fish by-products as a source of collagen can beneficially impact waste management. Collagen molecules in solution denature close to the upper limit of the physiological temperature or the maximum body temperature of the animal species from which the collagen is extracted [6]. Many researchers have focused on the practical utilization of marine animals to produce collagen [7]. Some concerned collagens from freshwater fish, such as carp [8,9] and grass carp [10]. However, relative lower denaturation temperatures, i.e., lower thermostability, have become one of the main limiting factors for the application of fish collagens, especially for those from marine fish. At present, the explanation for the reduced thermostability of collagens from fish is limited to the imino acid (hydroxyproline and proline) content of the samples. The denaturation temperatures of collagens increase with their imino acid content. Hydroxyproline may stabilize the triple helix by hydrogen-bonded water-bridges, as originally proposed by Ramachandran et al. [11].

The marine eel fish contains thick size of the skin, which is treated as waste of the home, fish shops, fish processing and preservations industries. In view of utilizing this wastes, the present study aimed to extract acid soluble collagen (ASC) and pepsin soluble collagen (PSC) from the skin waste of marine eel fish (*Evenchelys macrura*). In addition, the isolated collagen was characterized for its content and thermal stability.

## 2. Materials and methods

### 2.1. Materials and reagents

Acetic acid, sodium chloride (NaCl), Bovine serum albumin (BSA), trichloroacetic acid, pepsin, sodium dodecyl sulfate (SDS), acrylamide, ammonium persulfate, N,N,N',N'-tetramethyl ethylene diamine (TEMED) and Coomassie Brilliant Blue R-250 were purchased from Himedia Chemical Co. (India). The standard type IV collagen from human placenta (Sigma-Aldrich) and Achromopeptidase from *Achromobacter lyticus* were purchased from Sigma-Aldrich (EC 3.4.21.50, Mumbai, India). The standard molecular weight protein markers were purchased from GeNei (Bangalore, India). All Other chemicals and reagents used were of analytical grade.

### 2.2. Collection of sample

The outer skin wastes of eel fish were freshly collected from Parangipettai fish landing centre (Lat.11°29' N long.79°46' E), Tamil Nadu, south east coast of India and were brought to the laboratory (4 °C) and immediately washed with distilled water and stored at -20 °C until further used.

### 2.3. Proximate composition

The portions of the skin were removed from marine eel fish *E. macrura*, and after blending, the proximate composition was determined. The amount of moisture, fat, ash and protein content of skin were determined according to the AOAC [12] methods.

### 2.4. Isolation of collagen

#### 2.4.1. Pretreatment of outer skin waste

The collagen was extracted from the outer skin waste according to the method of Nagai and Suzuki [1] with suitable modification. All the experiments were performed at 4 °C unless otherwise indicated. Briefly, the outer skin was removed, cut into small pieces and defatted with 10 percent n-butyl alcohol (1:8) for 48 h, and washed with distilled water. Further, the skin was treated with 0.1 M NaOH to remove non-collagenous proteins for 3 days, washed with distilled water and lyophilized.

#### 2.4.2. Extraction of acid soluble collagen (ASC)

The lyophilized skin was soaked into 0.5 M acetic acid (1:3 weights per volume) for 3 days and the extracts were centrifuged at 20,000 × g for 1 h at 4 °C. The supernatant was collected and the residue was re-extracted by same procedure. The supernatant was mixed and desalting out by adding NaCl to a final concentration of 0.8 M and followed by precipitation of collagen by the addition of NaCl (final concentration of 2.3 M) at a neutral pH (0.05 M Tris-HCl, pH 7.5). The precipitates were collected and re-dissolved in 0.5 M acetic acid and dialyzed against 0.1 M acetic acid and distilled water for 2 days until the neutral pH was obtained. The dialyzed sample was lyophilized and referred to ASC.

#### 2.4.3. Extraction of pepsin soluble collagen (PSC)

The residue was thoroughly rinsed with distilled water, suspended in 0.5 M acetic acid and subjected to limited hydrolysis with 10 percent (weights per volume) pepsin (HiMedia, Mumbai, India) at 4 °C for 48 h. The viscous solution obtained was centrifuged at 20,000 × g for 1 h at 4 °C and the supernatant was dialyzed against 0.02 M sodium phosphate buffer (pH 7.2) for 3 days. Additionally, the sample was salted out by adding NaCl to a final concentration of 0.8 M followed by addition of NaCl to a final concentration of 2.3 M at neutral pH (0.05 M Tris-HCl, pH 7.5) for precipitation of collagen. The resultant precipitate was collected and re-dissolved in 0.5 M acetic acid. Finally, the resultant solution was dialyzed against 0.1 M acetic acid and distilled water then freeze-dried and referred to PSC.

#### 2.5. Quantification of ASC and PSC

The protein content of isolated collagen was determined by the method of Lowry et al. [13] using bovine serum albumin as a standard.

#### 2.6. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE)

Sodium-dodecyl-sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was performed by following the Laemmli [14] method using 12 percent acrylamide gel in the absence of β-mercaptoethanol (βME). After electrophoresis, the gel was stained with methanol: acetic acid: Coomassie brilliant blue R250 (80:20:0.3). The gels were destained by using de-staining solution containing the methanol, acetic acid and water (40:10:50). After destaining, the gels were plotted for molecular weight determination.

#### 2.7. Peptide mapping

The peptide mapping was determined through SDS-PAGE analysis performed by the method of Laemmli [14] using 15 percent gel. The lyophilized collagen samples (0.5 mg) were dissolved in 1 ml of 0.1 M sodium phosphate buffer (pH 7.2) containing 0.5 percent (weights per volume) SDS and heated at 100 °C for 5 min. After cooling the samples, the digestion was carried out at 37 °C for 30 min by adding 5 µg of achromopeptidase from *Achromobacter lyticus* (EC 3.4.21.50, Sigma-Aldrich, Mumbai, India) and the proteolysis was stopped by increasing temperature to 100 °C for 5 min. The peptide mapping was ascertained by comparing with standard type IV collagen and protein marker.

#### 2.8. Collagen solubility test

The optimum solubility test was carried out by dissolving in 0.5 M acetic acid at various pH and NaCl concentrations. Collagen samples were dissolved in 0.5 M acetic acid with gentle stirring at 4 °C for 12 h to obtain the final concentration of 3 and 6 mg/ml.

##### 2.8.1. Effect of pH

6 ml of collagen solution was transferred to the centrifuge tube and pH was adjusted with 6 N HCl to obtain final pH range between 1 and 10. The sample solution was made up to 10 ml with distilled water and adjusted the same pH. The solution was stirred gently for 30 min at 4 °C and centrifuged at 10,000 × g for 30 min at 4 °C. The relative solubility of collagen was calculated in comparison with pH rendering the highest solubility.

##### 2.8.2. Effect of NaCl

5 ml of collagen solution in 0.5 M acetic acid was mixed with 5 ml of cold NaCl in acetic acid of various concentrations (0–12 percent, w/v) to obtain final concentrations of 1–6 percent (weights per volume). The mixture was stirred gently at 4 °C for 30 min and centrifuged at 10,000 × g for 30 min at 4 °C. The relative solubility was calculated in comparison with that the salt concentration exhibiting highest solubility.

#### 2.9. Subunit composition

The separation of subunit composition was done by ion exchange column chromatography using DEAE (Diethyl amino ethyl) cellulose 52 (Sigma, India). The collagen sample was dissolved in 5 ml of 0.02 M sodium acetate buffer (pH 4.8) containing 6 M urea at 4 °C and denatured at 45 °C for 30 min. The denatured collagen was applied to DEAE cellulose 52 columns (1.5 cm × 8.0 cm) equilibrated with same buffer. Each subunit was eluted with a linear gradient of NaCl (0–0.2 M) at the flow rate of 0.4 ml/min. The collected fractions were examined for the presence of protein at 280 nm followed by Lowry et al. [13], pooled together and dialyzed.

#### 2.10. UV absorption spectrum

UV absorption spectrum of ASC and PSC was measured using a Shimadzu-UV-Spectrophotometer. The ASC and PSC (1 mg) were dissolved in 100 ml 0.02 M sodium acetate buffer (pH 4.8) containing 2 M urea. The UV spectrum was measured at wavelength between 190 and 400 nm at scan speed of 2 nm/s with an interval of 1 nm.

### 2.11. Determination of denaturation temperature ( $T_d$ )

The denaturation temperature ( $T_d$ ) was measured from changes in viscosity, using an Ostwald's type viscometer [10] with suitable modifications. Briefly, 20 ml of 0.03 percent collagen solution in 0.1 M acetic acid with 0.2 M sodium acetate buffer (pH 5.0) was used for viscosity measurements. Thermal determination curve was obtained by measuring the viscosity of the same solution at various temperatures (20–55 °C) at 15 min interval. The fractional viscosity at given temperature was determined as the temperature that the change in viscosity was half completed.

### 2.12. Circular dichroism measurement

Extracted collagen (5 mg) was diluted with 5 ml 0.1 M glycolic acid and the solution placed in a quartz cell with a path length of 0.1 or 1 cm. CD spectra measurements were performed by various temperatures at 25 °C, 26 °C, 27 °C, 28 °C, 29 °C, 30 °C, 32 °C, 34 °C, 36 °C, 38 °C, 40 °C, 42 °C, 44 °C and 45 °C for wavelengths of 190–280 nm at a scan speed of 2 cm/min. The mean molar ellipticity ( $\theta$ ) was calculated using the mean residue molecular weight and expressed in deg × cm<sup>2</sup>/dmol. The data were cumulated three times. In order to determine collagen denaturation temperatures, the rotatory angle at a fixed wavelength of 221 nm [ $\theta$ ]<sub>221</sub>, was measured as a function of temperature. The denaturation temperature,  $T_m$ , was determined as the temperature at which the change in ellipticity ( $\theta$ ) was half complete.

### 2.13. Amino acid profiling

The collagen samples were hydrolyzed under reduced pressure in 6 M HCl at 110 °C for 24 h. Amino acid composition was analyzed by using amino acid analyzer (Merck Hitachi LaChrome D-7000 HPLC System, Darmstadt, Germany). The hydrolysates were analyzed on a Hitachi LaChrome liquid chromatography system. The amino acid content is expressed as the number of residues/1000 residues.

### 2.14. Analysis of Fourier transform infrared spectroscopy (FTIR)

The collagen samples were analyzed using Fourier transform infrared (FTIR) spectrum. The lyophilized collagen samples (3 mg) were mixed with dried KBr (100 mg), ground in a mortar and pestle and subjected to a pressure of about  $5 \times 10^6$  Pa in an evacuated die to produce as 13 × 1 mm clear transparent disk. The absorption intensity of the peaks was calculated by the base-line method. The resultant spectra were analyzed using ORIGIN 8.0 software (Thermo Nicolet, USA).

### 2.15. Observation of scanning electron microscopy (SEM)

The morphological characteristics of the isolated collagen (ASC and PSC) were observed by JEOL JSM-5610LV Field Emission Gun SEM (Tokyo, Japan). Collagen samples were mounted on a standard SEM sample holder and fixed. The sample holder was used to prepare 20-s glow discharged carbon support adhesive films (tape) of 30 nm thickness and then smeared and coated with gold ion using auto fine coater. The samples were then introduced into specimen chamber of SEM and examined for surface morphology. The SEM observations were made at 20 kV accelerating voltage with a high vacuum (HV) mode and secondary electron image (SEI) was employed to scan the microscopic images of collagen matrixes. The average diameter of the collagens matrix was measured different arbitrary from SEM image. Samples were short-pulse coated with graphite to avoid damage due to overheating and analyzed on their fibrils side at increasing magnifications ( $\times 250$ –2000).

### 2.16. Statistical analyses

All the methods of extraction of collagen and analysis were replicated three times. The results were presented with mean ± standard deviations (SD).

## 3. Results and discussion

### 3.1. Proximate composition of eel fish skin

The proximate composition studies revealed that eel fish skin contained  $75.89 \pm 0.25$  percent moisture,  $90.05 \pm 0.23$  percent protein,  $1.23 \pm 0.02$  percent lipid and  $8.82 \pm 0.05$  percent ash on a dry weight basis which was slightly different that of the moisture, protein, fat and ash contents of brown backed toadfish skin contains 73.4, 90.3, 1.3 and 8.4 percent, respectively [15]. Moreover, the shark skin contained 61.96 percent moisture, 24.75 percent protein, 0.19 percent fat and 12.12 percent ash [16], and Nile perch skin contained 68.4 percent moisture, 21.6 percent protein, 6.8 percent fat and 6.0 percent ash [17].

In the present study, the protein content in eel fish skin was comparatively higher than the Nile perch skin and lower than the brown backed toadfish skin. Similarly, the moisture content was greater than the skin of balloon fish (62.23 percent) [18], shark skin (61.96 percent) [16], and scale of spotted golden goatfish (*Parupeneus heptacanthus*) (24.79 percent) [19]. Likewise, fat content in the *E. macrura* fish skin was comparatively lower than the Nile perch skin (5.5 percent) [17] and brown backed toad fish skin (0.05 percent) [15], but higher than the shark fish skin (0.4 percent) [16]. Conversely, the ash content of *E. macrura* fish skin was lower than the deep-sea redfish (39.4 percent) [20], and balloon fish (15.87 percent) [18], but little higher than that of brown backed toadfish (8.4 percent) [15], because of the eel fish skin covered without scales and small spine. After demineralization, ash content was about 8.80 percent, in which about 92 percent of inorganic matters were removed. Almost complete demineralization might cause the looser matrix of skin, which could be easier for collagen extraction.

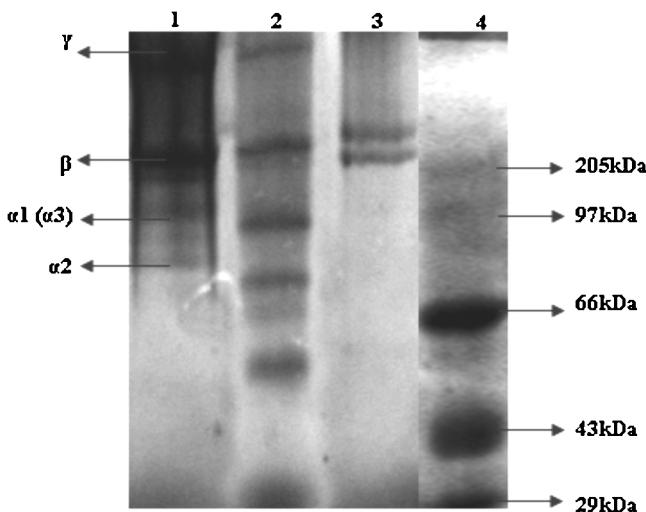
### 3.2. Yield of ASC and PSC from the skin of *E. macrura*

The final yield of ASC and PSC from the skin of marine eel fish was about 80 and 7.10 percent (dry weight basis) and 9 and 4.7 percent (wet weight basis), respectively. The eel fish skin was not completely solubilized by 0.5 M acetic acid extraction. In this result was in agreed with Jongjareonrak et al. [5,21] who reported that the incomplete solubilization of bigeye snapper (*Priacanthus macracanthus*) and brownstripe red snapper (*Lutjanus vitta*) skin in 0.5 M acetic acid. The result of the present study suggested that the collagen molecules in *E. macrura* fish skin were most likely cross-linked by covalent bonds through the condensation of aldehyde groups at the telopeptide region as well as the inter-molecular cross-linking, leading to a decrease in solubility of collagen [10,22]. 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 with acid. In the present study, the freeze dried ASC was found to be colorless fibril-like matters, whereas PSC was found to be a pale-black or grayish colored soft fiber due to pigment like ommochrome. Among the total collagen extracted, ASC (80 percent) based on extractable collagen weight was comparatively higher the PSC (7.10 percent). The greater content of ASC fraction in eel fish skin was in accordance with those reported in bigeye snapper (*Priacanthus macracanthus*) skin (85 percent) [21], brownstripe red snapper (*Lutjanus vitta*) skin was ASC (66 percent) [5]. However, the PSC fraction in eel skin was 2-fold higher than those obtained from those two species [5,21]. It is suggested that collagen with more inter-molecular cross-links is present to a greater extent in the skin of *E. macrura*, than in hake and bigeye snapper skin.

### 3.3. Gel electrophoresis patterns of collagen from the skin of *E. macrura*

The electrophoretic patterns revealed that both the ASC and PSC were consisting of  $\alpha_1$ - and  $\alpha_2$ -chains at a ratio of approximately 2:1 (Fig. 1) and high-molecular-weight components including  $\beta$  chain (dimers) and  $\gamma$  chain (trimers) components, as well as their cross-linked molecules. These results suggested that both ASC and PSC was most likely to be classified as type I collagen. Similarly, Jongjareonrak et al., also reported about the electrophoretic patterns of type I collagen from the skin of brownstripe red snapper [5]. The high MW cross-linked molecules in collagen was increases with animal age [22] and starving fish has more cross-linked collagen than well fed fish [23].

After digestion by pepsin, some  $\beta$ - and  $\gamma$ -components of ASC were cleaved into  $\alpha$ -components, as evidenced by the increasing



**Fig. 1.** SDS–polyacrylamide gel electrophoresis of Eel fish (*E. macrura*) and human placenta type IV collagen. Lane 1 contains ASC, Lane 2 contains PSC, Lane 3 contains Human placenta type IV collagen, Lane 4 contains Standard protein marker.

band intensity of the  $\alpha$ -chains. Similar electrophoretic protein patterns were found in ASC and PSC from the grass carp (*Ctenopharyngodon idella*) [10] and the pepsin cleaves the cross-link containing the telopeptide, and the  $\beta$ -chain is concomitantly converted to two  $\alpha$ -chains [24].

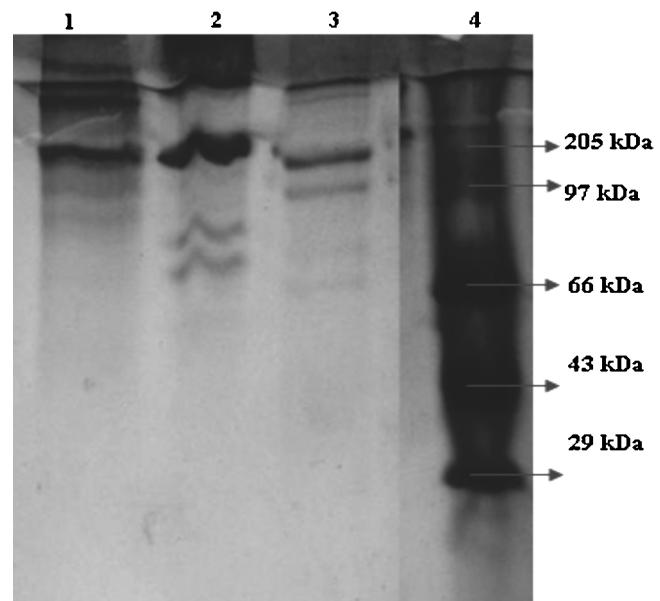
The type I collagen isolated from the present study consists of two  $\alpha_1$ - and one  $\alpha_2$ -chain as the major component ( $[\alpha_1]2\alpha_2$ ). Since the  $\alpha_3$ -chain has a molecular mass indistinguishable from  $\alpha_1$  chain and as it cannot be separated from  $\alpha_1$  chain under the electrophoretic conditions employed, the co-presence of  $\alpha_3$  with  $\alpha_1$  might be possible. The band intensity of  $\alpha_1$ -chain was 2-fold higher than that of  $\alpha_2$ -chain which consist of both ASC and PSC. The  $\alpha_2$  chain was found to be minor component of eel fish collagen with consisted of two  $\alpha_1$  and single  $\alpha_2$  chain. The results of the present study about  $\alpha_1$  and  $\alpha_2$  chain patterns were dissimilar to that of standard type IV collagen from human placenta (Lane 3) and similar to Nile perch collagen [17], and bigeye snapper [25].

#### 3.4. Peptide mapping of collagen from the skin of *E. macrura*

The electrophoretic pattern of denatured eel fish collagen suggested that the ASC and PSC gave very similar peptide maps; however, these patterns were different from standard type IV collagen (Fig. 2). After hydrolysis, the  $\alpha$ -chain and high molecular weight cross linked molecules of ASC and PSC from the skin of eel fish were degraded into small molecular weight peptides ranging from 384 to 73 kDa and 380 to 63 kDa, respectively. When compared to the other fish skin collagens of bigeye snapper [25] and Brownstripe red snapper [5] digested by V8 protease, the peptide maps were different. Peptide maps of collagens were reported to differ among from the other sources and species [26]. As a result, the pattern of the peptide fragment of eel fish skin collagen may be closely similar to that of mammalian skin.

#### 3.5. Effect of pH and NaCl concentration on collagen solubility

The effect of the pH and NaCl concentrations on the solubility of ASC and PSC extracted from the eel fish (*E. macrura*) skin were presented in Fig. 3. The results of the present study revealed that maximum stability of ASC and PSC was found to be at pH 4 and 3, respectively (Fig. 3A). Generally, these types of collagen were exhibited higher solubilization in acidic pH from 1 to 4 and the lesser in pH 7. In the solubility analysis, the ASC had higher solubility than



**Fig. 2.** Peptide maps of ASC and PSC from *E. macrura* skin waste collagens digested by *Achromopeptidase*. Lanes: 1, 2 and 3 contains standard collagen Type IV, ASC and PSC, Lane 4 contains protein markers.

that of the PSC with different pH. The solubility variation was due to ASC which consist of higher molecular weight protein than that of PSC. The dissimilarity in solubility of collagens with pH has been reported from big eye snapper skin and bone over pH ranges of 1–10 [25]. In the present study, we have exposed that the higher degree of molecular cross linking of ASC fraction which is due to the predominance of stronger bonds than PSC.

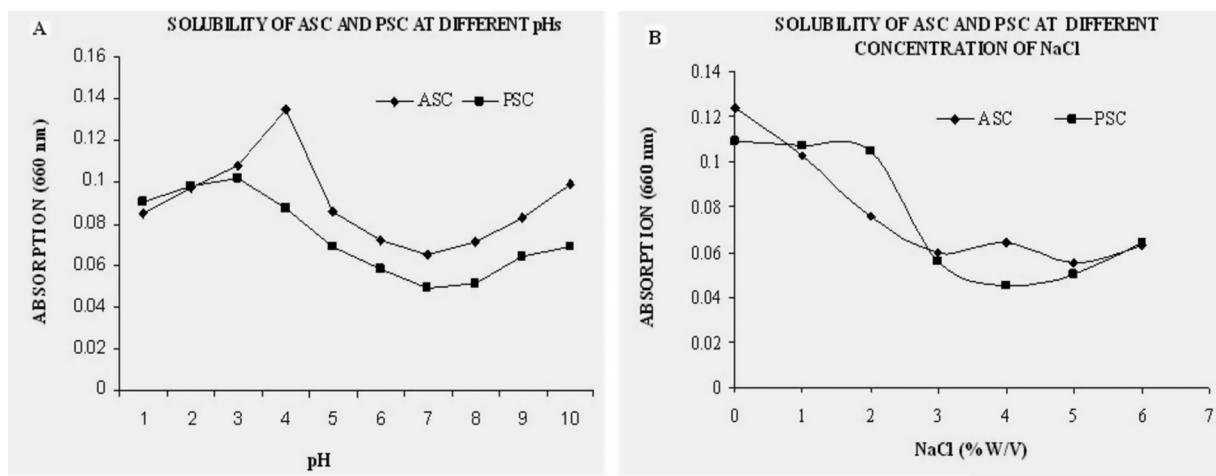
A drastic decrease in PSC solubility was observed with 3 percent NaCl or above. For PSC, solubility decreased up to 4 percent and it slightly increased at 6 percent NaCl. Whereas, the solubility of ASC was serially decreased up to 3 percent and rapidly reached the minimum of 4 percent and slightly decreased with 6 percent NaCl (Fig. 3B). The decrease in solubility of collagens could be described by the salting out phenomenon which occurred at relatively low NaCl concentration. An increase in ionic strength causes a reduction in protein solubility by an enhanced hydrophobic-hydrophobic interaction between protein chains, and competing for water of ionic salts, leading to the induced protein precipitation [27]. However, PSC exhibited a greater solubility than ASC at 2 percent NaCl concentrations which was due to the partial hydrolysis of high molecular weight cross-linked molecules by pepsin.

#### 3.6. Subunit composition of collagen from the skin of *E. macrura*

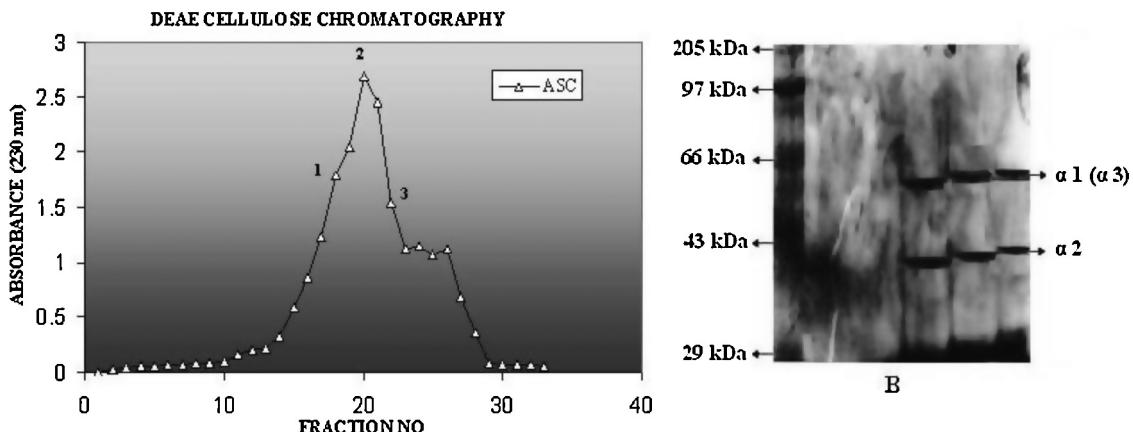
The subunit composition of ASC and PSC was determined by applying denatured collagen to DEAE-cellulose chromatography. As a result, a large peak was resolved having a shoulder on the right and it was supposed that the second and third fractions contain  $\alpha$ -chains as major components (Figs. 4 and 5) and small amount of  $\beta$ -chain (dimer) was observed in the PSC. Kimura et al. [28] suggested that two different heterotrimers of  $(\alpha_1)2, \alpha_2$  and  $\alpha_1, \alpha_2, \alpha_3$  were present in skin collagen of eel fish. The results of the present study evidenced about the occurrence of  $\alpha_3$  chain in eel fish skin collagen.

#### 3.7. UV absorption spectrum

The UV absorption spectrums of ASC and PSC at the wavelength ranges 190–400 nm were showed in Fig. 6(a) and (b). The results of the present study revealed that the amount of tyrosine in ASC and PSC from *E. macrura* were 3 residues per 1000 residues,



**Fig. 3.** Optimal solubility of both ASC and PSC from eel fish (*E. macrura*) skin waste in 0.5 M acetic acid at different pHs and NaCl concentration.

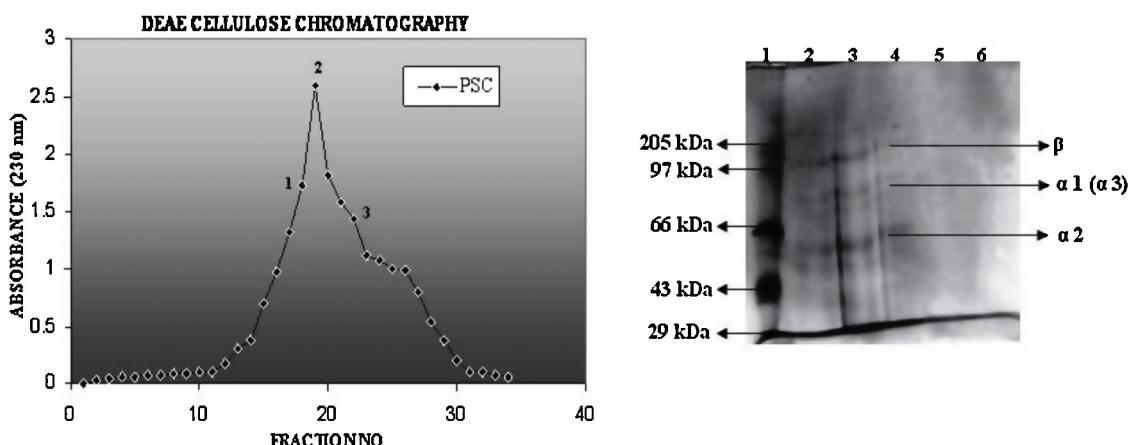


**Fig. 4.** Absorption capability of fractionated denatured ASC subunits at 230 nm in DEAE cellulose chromatography and the fractionation indicated by the numbers were examined by SDS-PAGE.

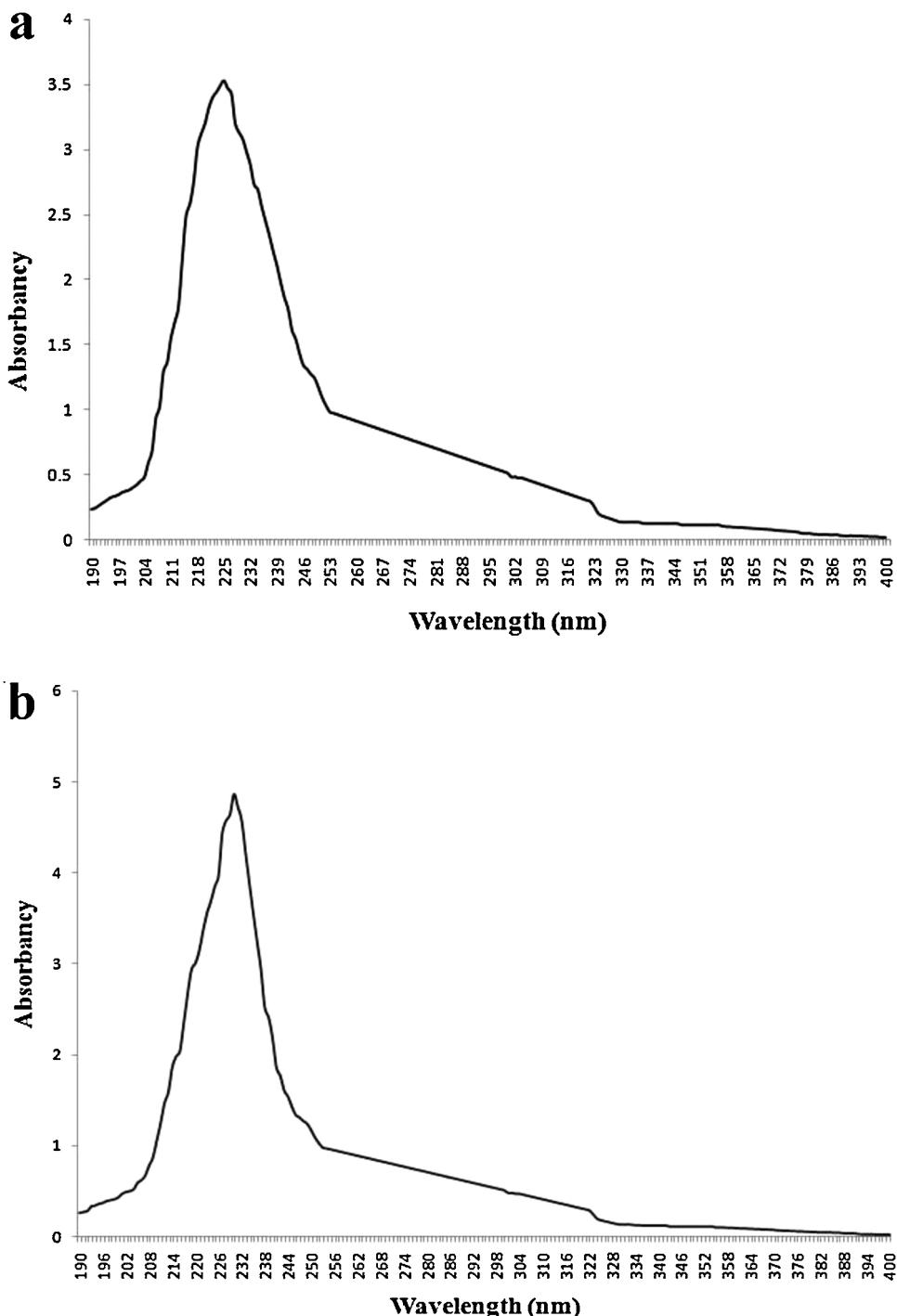
respectively. In the present study, ASC and PSC isolated from skin of *E. macrura* showed maximum absorption at 225 and 228 nm which is similar to the studies of Edwards et al. [29] which suggested that the groups C=O, –COOH, CONH<sub>2</sub> were accessible in polypeptides chains of collagen.

### 3.8. Thermal stability of collagen from the skin of *E. macrura*

The thermal denaturation temperature ( $T_d$ ) of eel fish skin collagen was calculated using thermal denaturation curve were shown in Fig. 7. ASC and PSC showed transition curves with maximum



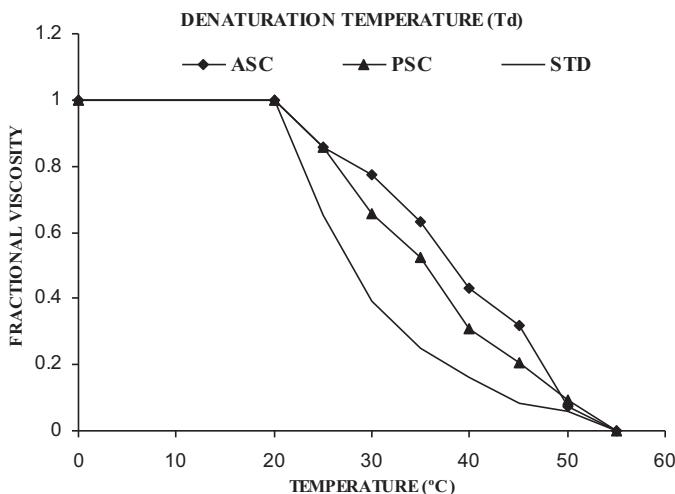
**Fig. 5.** Absorption capability of fractionated denatured PSC subunits at 230 nm in DEAE cellulose chromatography and the fractionation indicated by the numbers were examined by SDS-PAGE.



**Fig. 6.** (a) UV absorption spectrum of ASC from *E. macrura* skin. (b) UV absorption spectrum of PSC from *E. macrura* skin.

denaturation temperatures of 38.5 °C and 35.0 °C, respectively which is higher than that of type IV collagen from human placenta (28.5 °C). The present study suggested that the intramolecular hydrogen bonds stabilizing the triple helix structure of collagen might be disrupted to some levels in the presence of acetic acid, mainly due to the repulsion of collagen molecules in acidic solution [30]. Furthermore, a higher cross-linkage of marine eel fish skin collagen more likely contributed to the higher  $T_d$  of both ASC and PSC.  $T_d$  of collagen from the skin of *E. macrura* was much higher than that of ocellate puffer fish, 28 °C [31] and bigeye snapper (30.4 °C) [21], but was close to that of pig skin collagen (37 °C) [32].

The circular dichroism spectroscopy (CD) of ASC and PSC at the wavelengths of 190–280 nm were showed in Fig. 8(a) and (b) and the CD spectra of collagen over a temperature range of 25–45 °C. As a result, the CD curves showed a rotatory maximum at 230 nm, a minimum at 204 nm and a consistent crossover point (zero rotation) at about 252 nm, which was characteristic of the triple helical conformation of the protein [33,34]. Triple helical structure of collagen molecule is more stable with higher imino acid content as these facilitate intra and inter molecular crosslinking. Interestingly, the  $T_d$  of skin collagen of pig and calf (terrestrial mammals) is 37 °C and 40.8 °C, respectively [32], both having high iminoacid contents, whereas, cold-water fish collagens have low  $T_d$  since their imino



**Fig. 7.** Thermal denaturation curves of ASC and PSC from *E. macrura* skin and type IV collagen from human placenta as a standard.

acid contents are very low [35]. Thermal denaturation temperature of collagens from different sources has been direct correlation with the imino acid (proline and hydroxyproline) content [36]. There are also interesting consequences of variation in  $T_d$  with variation in temperature of their living environment. Therefore, this isolated type I collagen may find wide application due to its close denaturation temperature with mammalian collagen.

### 3.9. Amino acid composition of collagen from the skin of *E. macrura*

The amino acid composition of ASC and PSC extracted from the eel fish skin had similar amino acid profiles (Table 1) which was similar to the previously obtained from other aquatic animals [16,21,37]. In general, glycine is their major amino acid and one-third of total amino acid residues of collagen, because glycine could be found as being every third residue throughout the central region of the  $\alpha$ -chain other than the first 14 amino acid residues from the N-terminus and the first 10 amino acid residues from the C-terminus of the collagens [22]. In the present study,

**Table 1**

Amino acid composition of acid-soluble collagen and pepsin soluble collagen from *E. macrura* skin (residues per 1000 total amino acid residues).

Amino acids	Three letter	ASC	PSC
Alanine	Ala	125	117
Arginine	Arg	56	40
Asparagine	Asn	8	5
Aspartic acid	Asp	60	56
Cystine	Cys	12	15
Glutamic acid	Glu	80	83
Glycine	Gly	278	263
Histidine	His	3	4
Hydroxylysine	OH-Lys	4	6
Hydroxyproline	OH-Pro	94	98
Isoleucine	Ile	8	26
Leucine	Leu	9	13
Lysine	Lys	76	60
Methionine	Met	BDL	18
Phenylalanine	Phe	5	3
Proline	Pro	96	102
Serine	Ser	31	36
Threonine	Thr	24	22
Tryptophan	Trp	UND	2
Tyrosine	Tyr	3	3
Valine	Val	28	23

BDL, below detectable level; UND, undetectable.

both the collagens from the eel fish skin being found to have 26.3–27.8 percent glycine of total amino acid, and to be low in cystine, valine, leucine isoleucine, methionine, tyrosine and histidine, similar to other collagens [10,16]. Relatively high contents of alanine, proline, hydroxyproline and glutamic acid were observed and no tryptophan was detected, like other source of collagens [5,10,17,25,38].

The imino acid (proline + hydroxyproline) content of ASC and PSC was about 190 and 200 residues per 1000 residues, respectively, which was slightly lower than that of balloon fish collagen (197 and 203 residues per 1000 residues) [18], but much higher than that of cod skin collagen (179 and 174 residues per 1000 residues) [35]. The difference in imino acid content among the animals associated with different living environments of their sources, particularly habitat temperature [39]. Additionally, the imino acid content was reported to have a major influence on thermal stability of collagen [17]. 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. Hydroxylysine (4–6 residues per 1000 residues) was found in both ASC and PSC from eel fish skin. Hydroxylysine contributes to the formation and stabilization of cross links in the collagen [40].

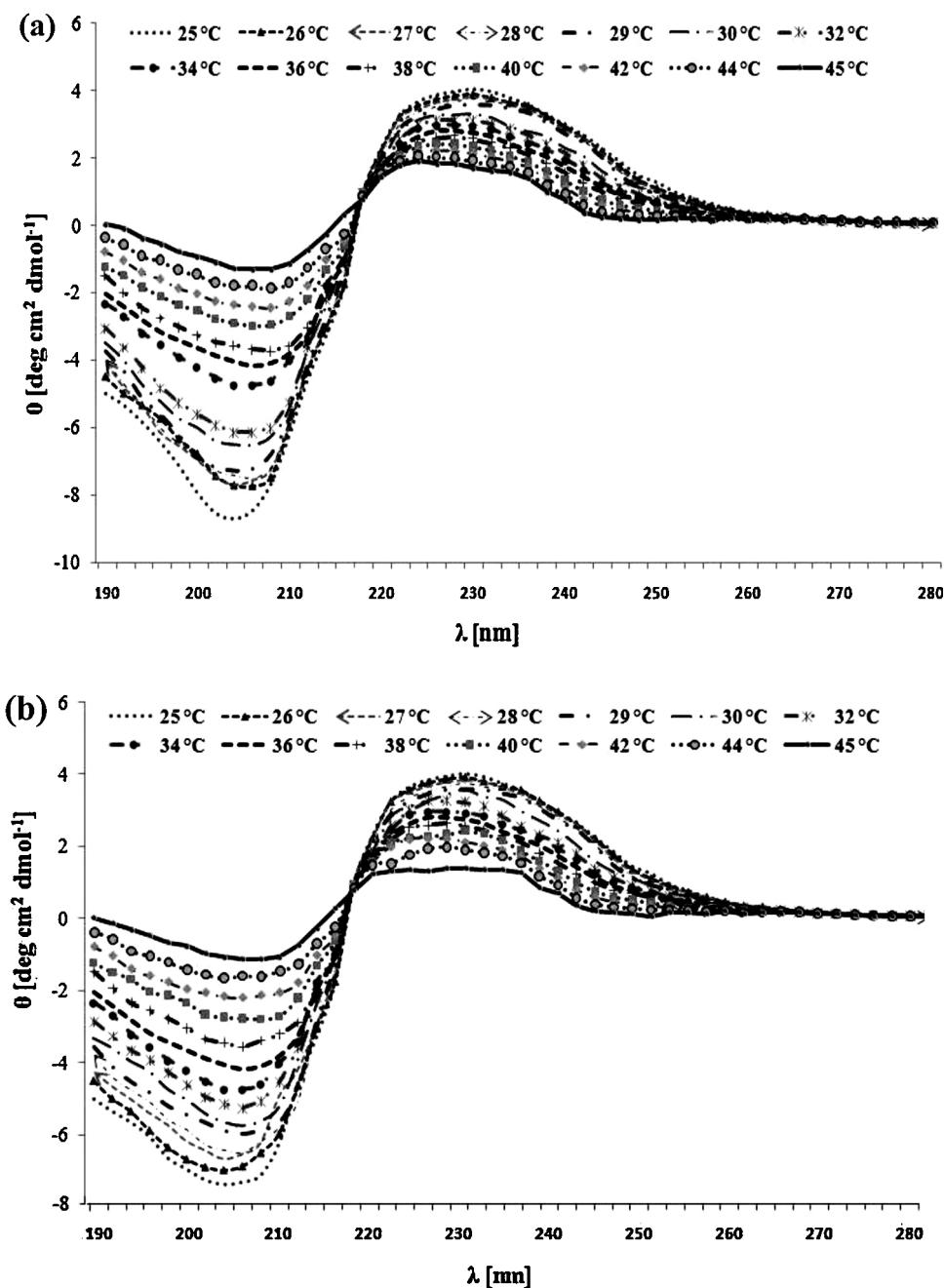
### 3.10. FTIR spectra of collagen from the skin of *E. macrura*

FTIR spectra of ASC and PSC from the eel fish (*E. macrura*) skin are shown in Fig. 9 and Table 2. The amide A band of ASC and PSC were found at 3421 and 3395 cm<sup>-1</sup>, respectively. This band is generally associated with the N–H stretching vibration and shows the existence of hydrogen bonds, probably with a carbonyl group of the peptide chain. A free N–H stretching vibration occurs in the range of 3400–3440 cm<sup>-1</sup>. The major peaks in the spectra of both ASC and PSC from the skin of eel fish (*E. macrura*) were similar to those of collagen from others fish species [17,20,37]. When the NH group of a peptide is involved in a hydrogen bond, the position is shifted to lower frequency, usually 3300 cm<sup>-1</sup> [41]. Amide B band of ASC and PSC was observed at 3079 and 3079 cm<sup>-1</sup> respectively, represent the asymmetrical stretch of CH<sub>2</sub> [17].

The sharp amide I band of ASC and PSC was observed at 1649 and 1653 cm<sup>-1</sup>, respectively. The amide I band, with characteristic frequencies in the range from 1600 to 1700 cm<sup>-1</sup>, was mainly associated with stretching vibrations of the carbonyl groups (C=O bond) along the polypeptide backbone [42], which is a sensitive marker of the peptide secondary structure [43]. 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. 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 1.17 between amide III and 1454 cm<sup>-1</sup> band of both collagens. Ratio of approximately 1 revealed the triple-helical structure of collagen [44]. The amide II of both collagens appeared at 1542–1541 cm<sup>-1</sup>, resulting from N–H bending vibration coupled with CN stretching vibration [45]. Thus, both ASC and PSC showed a similar secondary structure.

### 3.11. Morphological characterization of collagen from the skin of *E. macrura*

The morphological structures of the isolated collagens (ASC and PSC) were observed under SEM micro-photography with higher magnification (Fig. 10). The fibrillar structure was noted in both ASC and PSC and also mentioned that the porous three dimensional collagen fibril sponges were observed by SEM (Fig. 10).

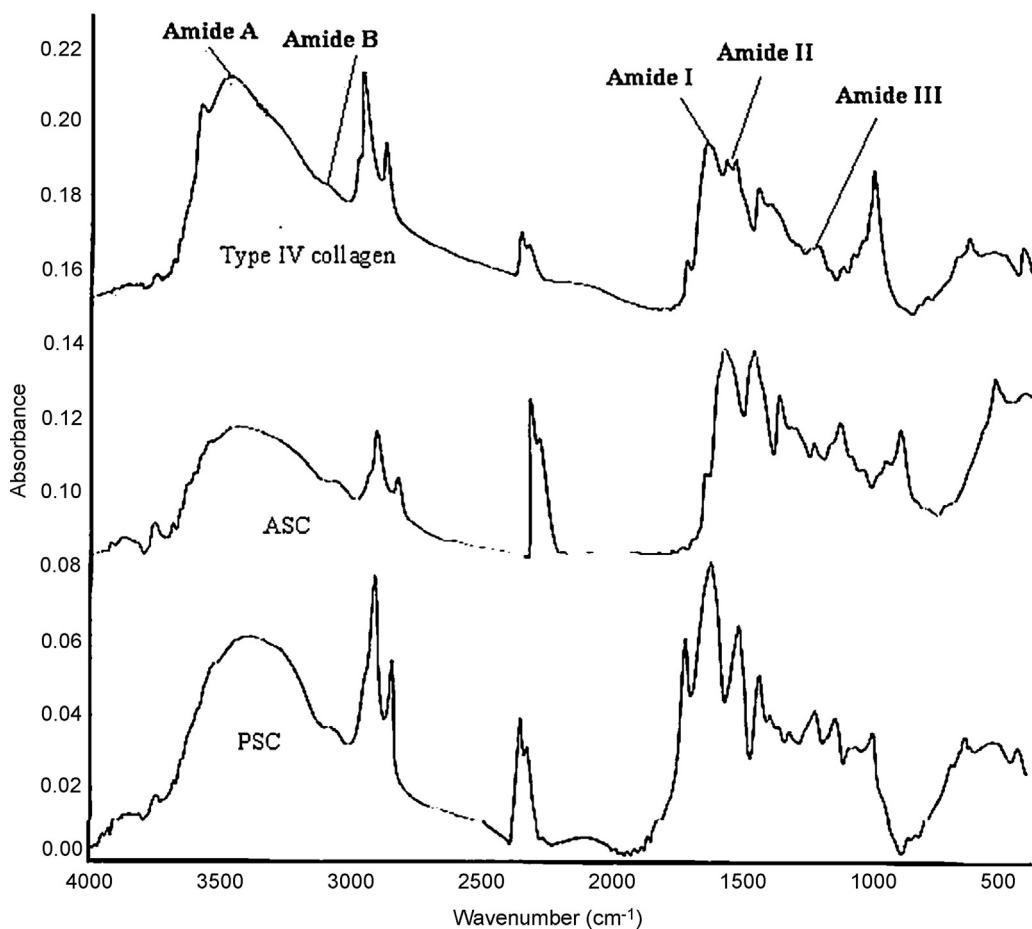


**Fig. 8.** (a) CD spectra of the ASC from outer skin of the Eel fish measured at temperatures 25–45 °C. (b) CD spectra of the PSC from outer skin of the Eel fish measured at temperatures 25–45 °C.

The morphological structures of ASC and PSC were fibrillar in nature. However, the fibril width range 1 μm of the ASC was lower than that of pepsin soluble collagen. The width of collagen fibrillar tubes was uniform in size (~1 μm) and PSC had nodular-like structures with tubular in nature. The results of the present study revealed that SEM images has been confirmed that both ASC and PSC showed the cross-section in contact with the mold that exposes an inter-connected network pore configuration with a pore size of 90–250 μm. The regular porous structure was clearly visible in ASC when compared to PSC. The pore size of collagen was increased at higher water content during preparation and the size was moderate for in vivo studies and comparatively similar to the previous reports [46]. In addition, the collagen extracted from the bone of horse mackerel and croaker could be used as biofilm or scaffold

that can be used for wound healing purposes because, for wound management, freeze-dried collagen sponges are frequently placed onto wounds without cells [47].

The epineurium consists of thick bundles of collagen fibrils measuring about 10–20 μm width; they were wavy and ran slightly, obliquely to the nerve axis. Between these collagen bundles, very coarse meshwork of randomly oriented collagen fibrils was present. The outer one consisted of longitudinally oriented bundles of about 1–3 μm in width. The collagen fibril arrangement described above may protect the nerve fibers against external forces. Although these collagen fibrils formed bundles, they were not parallel but were entangled in individual bundles. These collagen bundles varied in width and thickness, often gave off branches, and intertwined with each other. Pore size, porosity and surface areas are widely



**Fig. 9.** Fourier transforms infrared spectra of standard type IV collagen from human placenta, acid soluble collagen (ASC) and pepsin soluble collagen (PSC) from the skin of *E. macrura*.

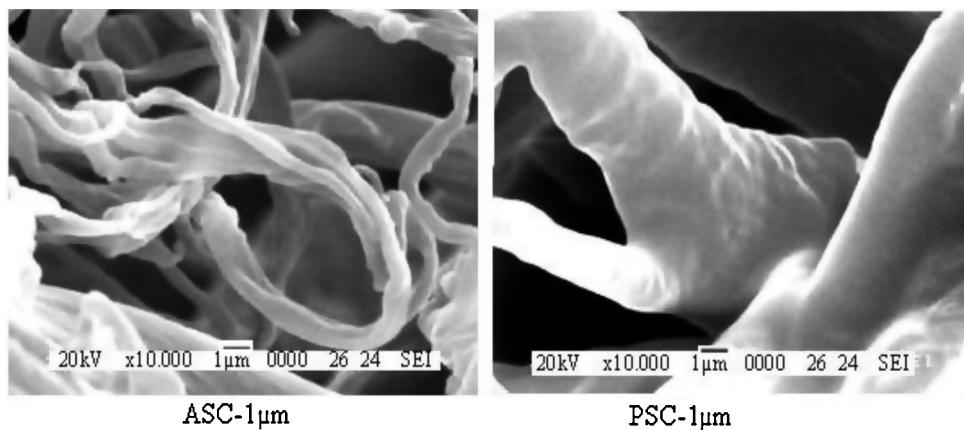
recognized as important parameters for a biomaterial to understand their biomedical importance [48]. In addition, other architectural features such as pore shape, pore wall morphology and interconnectivity have also suggested for cell seeding, migration, growth, mass transport, gene expression, and new tissue

formation. Generally, uniform and regular network structure of collagen as drug carrier is propitious, for well-proportioned distribution for other drugs [10]. Based on the forgoing account, the isolated marine eel fish skin collagen could be used as an appropriate drug carrier system.

**Table 2**

General peak assignments of the FTIR spectra consist of control standard (STD) type IV collagen, ASC and PSC from eel fish *E. macrura* skin.

Peak wave numbers cm <sup>-1</sup>	Control (standard type IV collagen)	Acid soluble collagen	Pepsin soluble collagen	Region peak assignments
3545	3529	—	—	N—H asymmetric stretching of primary amide (Monomer)
3421	3421	3395	—	Amid A: mainly N—H stretching of proteins
2959	3079	3079	—	Amid B: CH <sub>3</sub> -asymmetric stretch
2922	2923	2924	—	Amid B: CH <sub>2</sub> -asymmetric stretch
2852	2853	2854	—	CH <sub>3</sub> -symmetric stretch: mainly proteins
1743	1743	1742	—	Carbonyl C=O stretch: lipids
1644	1649	1653	—	Amide I: C=O stretching of proteins
1578	1542	1541	—	Amide II: N—H bending/C—N stretching of proteins
1541	—	—	—	Amide II: N—H bending/C—N stretching of proteins
1461	1458	1458	—	CH <sub>3</sub> -asymmetric bending: mainly lipids
—	1339	1338	—	COO-symmetric stretch: mainly phospholipids
1249	1241	1243	—	Amide III ( $\beta$ -sheet, protein)
1156	—	1165	—	CO—O—C asymmetric stretch: glycogen and nucleic acids
—	1079	—	—	PO <sub>2</sub> -symmetric stretching: mainly nucleic acids
1020	1022	1022	—	C—O stretching/C—O banding of the C—O—H carbohydrates
833	—	—	—	Out of plane breathing Tyr; PO <sub>2</sub> - asymmetric stretch DNA (B-form)
723	—	723	—	Adenine
670	669	670	—	CH <sub>2</sub> banding, carbohydrates, proteins, and lipids (sterols of fatty acids)
—	562	559	—	N—H out of plane banding
467	467	467	—	Out of plane ring banding



**Fig. 10.** Scanning electron microscopic plates exhibiting the fibrillar structure of ASC and PSC isolated from *E. macrura* (1  $\mu\text{m}$  resolution).

## 4. Conclusion

A great quantity of collagen (ASC and PSC) could be extracted from marine eel (*E. macrura*) fish skin with yield of 80 and 7.10 percent (dry weight basis), respectively. The collagen extract consists of two  $\alpha$ -chains ( $\alpha 1$  and  $\alpha 2$ ) and were characterized as type I collagen. In the present study, the biochemical analyses, such as protein quantification, SDS-PAGE, followed by peptide mapping and solubility effect of pH and NaCl test, on collagen extracted by the acetic acids based method could be used to extraction of higher yields of type I collagen from eel fish skin waste. The ASC and PSC showed high solubility at acidic pH and lost solubility when the NaCl concentrations were increased. On the basis of our results, we have suggested that the determination of the thermal stability ( $T_d$ ) of isolated collagen was relatively higher (38.5 °C and 35.0 °C) than that of other fish collagens reported elsewhere may be due to presence of high imino acid content. These collagens were type I mainly, with similar amino acid composition, and maintained their triple helical structures well, with slight differences in terms of thermal stability and molecular structure. FTIR spectra of both ASC and PSC were quite similar in terms of their primary and secondary structures. The isolated type I collagen may serve as an attractive alternative to mammalian collagen for biomedical and pharmaceutical applications owing to its fair closeness in  $T_d$  with mammalian collagen. Therefore, there is a prospect of using the fish processing waste as an alternative source of collagen; which otherwise may cause serious environmental pollution.

## Conflict of interest statement

The author declares that there are no conflicts of interest.

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