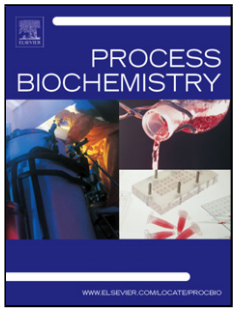

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Characterization of Pacific cod (*Gadus macrocephalus*) skin collagen and fabrication of collagen sponge as a good biocompatible biomedical material

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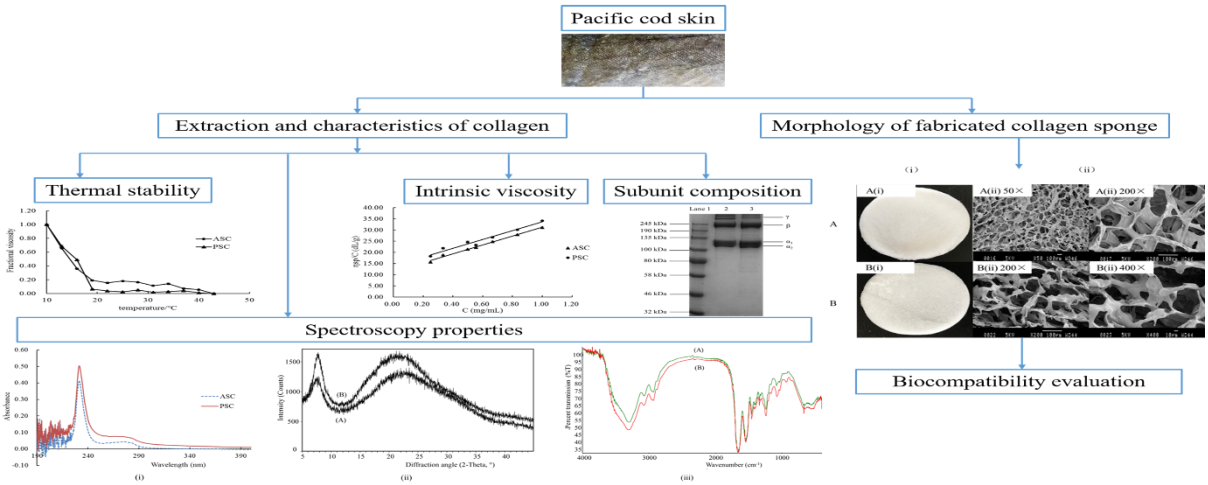
Graphical Abstract



Highlights

1. Pacific cod skin is an excellent collagen source replacing mammalian sources.
2. Both are typical type I collagen with α chain composition of $(\alpha_1)_2\alpha_2$.
3. Extracted collagens maintain their native and intact triple helical structure.
4. No notable difference in physicochemical properties between ASC and PSC.
5. Pacific cod skin collagen has a good blood biocompatibility.

Abstract: Acid-soluble collagen (ASC) and pepsin-soluble collagen (PSC) were extracted from Pacific cod (*Gadus macrocephalus*) skin and preliminarily purified via salting-out. Physicochemical properties of both collagen were determined. Amino acid composition analysis indicated that the contents of proline and hydroxyproline

were 157 and 159 residues/1000 residues, respectively. And the proline hydroxylation rates were 40.8% for ASC and 41.5% for PSC. Denaturation temperatures (Td) measured with Ubbelohde viscometer were 14.5°C and 16°C, respectively, lower than that of mammals. Moreover, the analysis results of intrinsic viscosity, SDS-PAGE, Ultraviolet (UV) absorption, X-ray diffraction (XRD) and Fourier transform infrared spectroscopy (FTIR) demonstrated that ASC and PSC were triple-helical type I collagens with α chain composition of $(\alpha_1)_2\alpha_2$. Meanwhile, the scanning electron microscopy (SEM) and the hemolysis assay *in vitro* indicated that collagen sponge fabricated by freeze-drying technology had uniform and porous structure and good biocompatibility. So it can be applied in biomedical materials field.

Keywords: Collagen; Characterization; Collagen sponge; Biocompatibility; Biomedical material

1. Introduction

Collagen is the most abundant protein as well as a primary component of the extracellular matrix (ECM) [1]. So far, about 28 types of collagen have been

recognized in multifarious tissues [2]. Collagen consists of three polypeptide chains and twists into a triple-helix structure. Of all types, type I collagen is the most common type.

Nowadays type I collagen is widely applied in biomedical raw materials thanks to its favorable biological features, such as biocompatibility, biodegradability and weak antigenicity [3, 4]. Among the natural biological macromolecules, collagen is a perfect adsorbable ingredient of hemostatic agent and wound dressing [5]. An increasing number of studies have found that collagen can also be used in drug delivery [6] and tissue engineering [7-9]. Collagen sponge can be better applied in hemostasis and wound healing owing to its excellent characteristics including absorption of tissue exudates, good gas permeability and promotion of the growth of epithelial cells and the generation of granulation tissue.

The major sources of collagen were bovine tendon and porcine skin in previous researches. However, because of the prevalence of a variety of diseases including foot-and-mouth disease (FMD), bovine spongiform encephalopathy (BSE), transmissible spongiform encephalopathy (TSE) [10] and religious barriers, researchers have considered marine by-products as alternative sources of collagen [11, 12]. Pacific cod is a main commercial fish with a steadily increased output. Most of these are processed into fillets, leaving behind more than 30 thousand tons of fish skins per year. Fish skins are rich in collagen, constituting 70%–80% of the dry matter [13], so it is significant to turn by-products into value-added collagen. There were some research on characterizations of collagen from walleye pollock [14], Baltic cod

[15, 16] and gelatin peptides from Pacific cod skin [17]. However, there were few systematic comparative studies of characteristics between acid-soluble collagen (ASC) and pepsin-soluble collagen (PSC) from Pacific cod (*Gadus macrocephalus*) skin. And the application of Pacific cod skin collagen in biomedical materials field and its biocompatibility were also insufficient in research. So the properties of ASC and PSC were compared in this paper, and further application in biomedical materials field was studied.

2. Materials and methods

2.1. Materials and reagents

The frozen Pacific cod skins were obtained from Qingdao Long Harmony Food Co., Ltd (Qingdao, China), and stored at -20°C until used.

Male Wistar rats (200–220 g weight) were obtained from Jinan Pengyue Laboratory Animal Breeding Co., Ltd (Jinan, China).

Pepsin (1: 3000 U) was obtained from Beijing Solarbio Science & Technology Co., Ltd (Beijing, China). The protein marker for electrophoresis was provided by New England Biolabs Inc. (USA). All other reagents were of analytical grade.

2.2. Extraction of ASC

Pacific cod skins were cleaned up, removed residual fat and flesh of fish and cut into small pieces (approximately 5×5 mm). The following procedures were performed on ice or at 4°C as mentioned by Senaratne et al. [18] with some modifications. The pieces of fish skins were soaked into 20 volumes (v/m) of 0.1 M NaOH with a

constant stirring (78-1, Danrui Experimental Instrument Equipment Co., China) for 24 h to remove non-collagenous impurities. The alkali-treated fish skins were washed repeatedly to neutral and stirred gently in 0.5 M acetic acid with solid/solvent ratio of 1: 60 (w/v) to extract for 48 h. Then the supernatants after centrifugation (2-16 KL, Sigma, USA) at 9600 g for 30 min at 4°C were salted-out by the addition of grinded NaCl to a final concentration of 0.9 M. The mixture was left overnight and centrifuged at 8600 g for 30 min at 4°C. The resulting precipitate was dissolved in 0.5 M acetic acid. Then the solution was dialyzed against 0.1 M acetic acid for 24 h followed by deionized water for 48 h. Both dialysates were renewed every 8 h. The purified ASC was lyophilized (VSP-62, Marin Christ Co., Germany), sealed in polythene bags and stored in a dryer for subsequent analysis.

2.3. Extraction of PSC

The extraction procedures of PSC were similar to ASC. The fish skins after alkali treatment were extracted with 60 volumes (v/w) of 0.5 M acetic acid in the presence of 0.5% (w/w) pepsin. The crude extract was depurated via salting-out. Then the resulting precipitate after centrifugation was re-dissolved in 0.5 M acetic acid and dialyzed against 0.02 M Na_2HPO_4 for 48 h to inactivate pepsin followed by 0.1 M acetic acid for 24 h and deionized water for 48 h. The lyophilized PSC was sealed and used for subsequent analysis.

2.4. Amino acid analysis

The ASC and PSC samples were hydrolyzed in 6 M HCl for 24 h at 110°C under reduced pressure. The amino acid composition of hydrolysates was determined with an amino acid analyzer (L-8900, Hitachi, Japan).

For hydroxyproline, ASC and PSC samples were determined in accordance with the method of Woessner [19] with slight modification. The samples (approximately 5 mg) were weighed accurately, sealed into ampoule bottles and hydrolyzed in 1 mL of 6 M HCl at 130°C for 4 h. Then the hydrolysates were cooled to room temperature, transferred thoroughly into 100 mL volumetric flasks and adjusted to alkaline with phenol phthalein indicator. The L-hydroxyproline standard was dissolved and diluted with 0.001 M HCl to 10 μg/mL to prepare a standard solution series. Then 1 mL sample solution with the addition of 1.5 mL absolute ethanol and the L-hydroxyproline standard solution series were mixed with 1 mL chloramine T and the mixtures were stood at room temperature for 20 min. After adding 1 mL perchloric acid to each tube to destroy chloramine T for 5 min, 1 mL para-dimethylaminobenzaldehyde was joined, shook rapidly, placed in a 60°C water bath and allowed to stand for 20 min. The reacted solutions were cooled to room temperature with running water and measured at 560 nm.

2.5. Determination of denaturation temperature (Td)

The denaturation temperature of ASC and PSC was measured with Ubbelohde viscometer method mentioned by Zhang et al. [20] with some modifications. Viscosity was determined at different temperatures from 10°C to 43°C at an interval

of 3°C. The corresponding fractional viscosity at a specific temperature was calculated by the following equation:

Fractional viscosity= ($\eta_{sp}(T)$-η_{sp}(43°C))/(η_{sp}(10°C)- η_{sp}(43°C)) (1)

$\eta_{sp} = (t-t_0)/t_0=\eta_r -1$ (2)

$\eta_r = t/t_0$ (3)

where η_{sp} is specific viscosity, η_r is relative viscosity, t and t_0 are the time of collagen samples (0.5 mg/mL) and solvent (0.1 M acetic acid) passing through capillary at a specific temperature, respectively. The Td of ASC and PSC was the temperature where fractional viscosity was 0.5 in thermal determination curve.

2.6. Determination of intrinsic viscosity

The intrinsic viscosity of ASC and PSC was measured with Ubbelohde viscometer method. The lyophilized collagen samples were dissolved and diluted to a series of concentrations of 1, 5/6, 2/3, 5/9, 1/2, 1/3, 1/4 mg/mL with 0.1 M acetic acid, respectively. The time of collagen solutions passing through capillary at 10°C constant temperature was recorded. The intrinsic viscosity ([η]) was determined by the formula:

$$[\eta]=\lim_{c\to 0} \eta sp/c \quad (4)$$

where c is the concentration of collagen solutions, and the intrinsic viscosity ([η], dL/g) is the intercept of η_{sp}/c plotted against c when $c\to 0$.

2.7. *Ultraviolet (UV) absorption spectra*

The ASC and PSC were dissolved in 0.5 M acetic acid to a concentration of 0.5 mg/mL and centrifuged at 10600 g for 20 min at 4°C. The spectra were recorded using an ultraviolet and visible spectrophotometer (UV-2802, Unico, China) from the wavelength of 190 to 400 nm at a medium speed scanning rate with an interval of 0.2 nm.

2.8. *X-ray diffraction (XRD)*

The X-ray diffraction (X Pert Pro MPD, Anton Paar, Austria) of ASC and PSC samples was analyzed with reference to the method reported by Giraud et al. [21]. The wavelength of radiation was $\lambda_{Cuk\alpha 1}$=1.5406 Å. It scanned the 2θ interval between 4° and 45° by a step of 0.02° at an angular speed of 4° min^{-1}.

2.9. *Fourier transforms infrared spectroscopy (FTIR)*

1 mg ASC and PSC samples mixed with approximately 100 mg dried potassium bromide (KBr, spectrum pure) were ground and pressed into relatively transparent sheets under dry conditions. The FTIR spectra were recorded from 4000 to 500 cm^{-1} at a data acquisition rate of 2 cm^{-1} with scanning times of 64 by an infrared spectrophotometer (iS 10, Thermo-Nicolet Co., USA). The peak wavelength values were analyzed with the OMNIC 8.0 software.

2.10. *Sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE)*

SDS-PAGE was based on the method mentioned by Laemmli [22] using 7.5% resolving gel and 5% stacking gel. 1 mg/mL collagen samples in 0.5 M acetic acid

were neutralized and centrifuged at 9200 g for 30 min at 4°C. Then the resulting supernatants were mingled with loading buffer in the presence of beta-mercaptoethanol, boiled for 5 min, and centrifuged at 2300 g for 5 min. Then 15 μL of supernatants were loaded into each path, along with 10 μL of the protein marker. The electrophoresis was carried out at a constant voltage of 80 V for about 20 min followed by 120 V. After electrophoresis, gel was stained in Coomassie blue R-250 (0.1%, w/v) staining solution for 1 h and discolored overnight.

2.11. Fabrication of collagen sponge

10 mg/mL collagen solutions in 0.5 M acetic acid were dialyzed against deionized water at 4°C for 3 d. Air bubbles were removed by means of vacuum and centrifugation. The collagen solution was poured into six-well plates and then freeze-dried. The pre-freezing procedure was implemented at -40°C for 34 h and then freeze-dried at -38°C under a vacuum of 0.06 mbar.

2.12. Scanning electron microscopy (SEM)

The surface and cross section of collagen sponge were pasted on an aluminum plate and placed inside an ion coater (IB 3, Eiko, Japan) for gold sputtering. Then the images of sputtered specimens were observed with scanning electron microscope (JSM-840, JEOL, Japan) at a voltage of 5 kV.

2.13. Blood compatibility evaluation

Blood compatibility was evaluated by hemolysis assay *in vitro* on the basis of the method mentioned by ASTM F 756-00, 2000 with own modifications. The collagen

sponge was cut into 1 cm×1 cm, rinsed in 10 mL of normal saline and pre-heated at 37℃ for 30 min. Anticoagulated whole blood was obtained from Wistar rats and diluted in a ratio of 4: 5 with normal saline. Then 0.2 mL of diluted anticoagulated blood was added and maintained at 37℃ for 60 min. Subsequently, the supernatant after centrifugation at 1000 g for 5 min was used to measure absorbance of hemoglobin at 540 nm. 100% hemolysis, a positive control, was operated by adding 0.2 mL of diluted anticoagulated blood into 10 mL of deionized water and 0% hemolysis, a negative control, was operated by adding 0.2 mL of diluted anticoagulated blood into 10 mL of normal saline. Hemolysis ratio (HR) was calculated by the following equation:

$$HR\ (\%) = (OD_s\text{-}OD_n)/(OD_p\text{-}OD_n) \times 100 \quad (5)$$

where OD_s, OD_n, OD_p are the absorbance of samples, negative control and positive control, respectively.

2.14. Statistical analysis

Three replicates were measured for each sample. All quantitative data were shown as the average ± standard deviation.

3. Results and discussion

3.1. Amino acid composition and thermal denaturation temperature of collagen

The amino acid composition of ASC and PSC, expressed as residues per 1000 total amino acids, was showed in Table 1. Collagen was rich in glycine, alanine, proline, and hydroxyproline, with few cysteine and tyrosine. The glycine was the most

abundant amino acid, accounting for 33.7%, 35.5%, respectively, which was similar to other sources of collagen [23-25]. This was consistent with the fact that glycine was the smallest amino acid and the $(Gly\text{-}X\text{-}Y)_n$ repeated in collagen polypeptide chains to assembly into a triple helix structure. No tryptophan existed in collagen.

The contents of proline and hydroxyproline were 157 and 159 residues/1000 residues, respectively, which was similar to that of cod skin collagen (154 residues/1000 residues) reported by Duan et al. [26], but lower than that of carp skin (192 residues/1000 residues) [26], porcine skin (220 residues/1000 residues) [27] and bovine skin (215 residues/1000 residues) [28]. The contents of proline and hydroxyproline were correlated to species and their living temperature [29]. The pyrrolidine rings of proline and hydroxyproline contribute to strengthen the triple helix structure of collagen [30]. The hydroxyproline content of ASC and PSC were 64 and 66 residues/1000 residues, respectively. Hydroxyproline is indispensable to form an intramolecular hydrogen bond and plays a key role in collagen thermal stability. The proline hydroxylation rate (hydroxyproline content/ the contents of proline and hydroxyproline) were 40.8% for ASC and 41.5% for PSC, higher than that of ocellate puffer fish (39%) [11], but lower than that of channel catfish (43%) [31] and cuttlefish (48%) [32].

The determination of denaturation temperature (Td) was on the basis of viscosity variation. The transition curve of ASC was similar to PSC, and the fractional viscosity decreased rapidly from 10°C to 20°C. But it was almost stable at temperatures above 20°C. The Td values of ASC and PSC were approximately 14.5°C and 16°C,

respectively (Fig. 1), similar to that of Alaskan pollack (16.8°C) [33], but lower than that of Nile tilapia skin (35.2°C, 34.5°C) [23], *O. niloticas* (28°C) [34] and carp skin (31.7°C) [35]. Td values were relevant to temperatures of species survival environment. The thermal stability of collagen was positively correlated to the contents of proline and hydroxyproline. The results were also consistent with that of amino acid composition mentioned above. In general, higher contents of proline and hydroxyproline and proline hydroxylation rate resulted in a better thermal stability and a higher Td value.

3.2. Intrinsic viscosity of ASC and PSC

The intrinsic viscosity of natural triple-helical collagen is 11.5 dL/g or 13.4±0.9 dL/g, a characteristic of rod-shaped molecules [36], whereas random coil conformation ranges from 0.1 to 1.5 dL/g and compressed spherical particles are limited to 0.05–0.2 dL/g. It is in accordance with the results that the intrinsic viscosity of gelatin is 0.5174 dL/g and collagen hydrolysates is 0.0641 dL/g [37].

The intrinsic viscosity curves of ASC and PSC were as shown in Fig. 2. The linear equations are given by $y=19.806x+11.436$ ($R^2=0.9921$) and $y=19.658x+13.967$ ($R^2=0.9661$), respectively. The intercept is just the intrinsic viscosity of ASC (11.436 dL/g) and PSC (13.967 dL/g) when the concentration is zero. These results are in line with studies reported by Montero et al. [38], who indicated that rod-shaped collagen isolated with acetic acid was non-denatured. These results also showed that the ASC and PSC extracted with the method mentioned above retained their native triple helix structure.

3.3. Spectroscopy properties

The spectroscopy properties of ASC and PSC from Pacific cod skin were expressed by UV absorption spectra, X-ray diffraction (XRD) and Fourier transform infrared spectroscopy (FTIR) (Fig. 3).

From Fig. 3 (i), both ASC and PSC from Pacific cod skin exhibited a maximum UV absorption at 231 nm, which was in accordance with type I collagen, similar to that of channel catfish skin (232 nm) [31]. The UV absorption was possibly related to –COOH, $CONH_2$ groups in polypeptide chains and n→π* transition of C=O in peptide bond [39]. It was also in accordance with the result that there was few tyrosine and phenylalanine in the amino acid composition of collagen (Table 1). Tyrosine and phenylalanine were sensitive chromophores contributing to the ultraviolet absorption at 283 nm and 251 nm [40], where ASC and PSC had no obvious absorption. The characterization of ultraviolet absorption also showed that collagen extracted with the method described above had high purity without many other proteins.

As shown in Fig. 3 (ii), the XRD diagrams of both ASC and PSC had two peaks, which was a characteristic of collagen. The corresponding diffraction angles (2θ) at peaks were 7.59°, 22.44° for ASC, and 7.70°, 21.79° for PSC. The Bragg equation was described to calculate the minimum of the repeated interval as followed: d (Å) = λ/2sin θ, where λ is the X-ray wavelength (its value is 1.54 Å), and θ is referred to as the Bragg diffraction angle [41].The first sharp peak was related to the triple-helical structure of collagen, and its corresponding d values were 11.63 Å for ASC and 11.47

Å for PSC, reflecting the distance between molecular chains. The d values of the second relatively broad peak were 3.96 Å and 4.07 Å, respectively, indicating the distance between skeletons. These results were close to that of carp scale collagen [20]. These data were consistent with the diameter of a single left-handed helix chain and triple-helical collagen molecule. In general, the ASC and PSC extracted from Pacific cod skin were non-denatured with native triple helix conformation.

The characteristic absorption peaks of FTIR spectra were shown in Fig. 3 (iii). From the spectra, ASC was almost identical to PSC, which was also in agreement with the findings reported by Zhang et al. [42]. The range of a free N-H stretching vibration was 3400–3440 cm^{-1}, while it could shift near to 3300 cm^{-1} thanks to the existence of hydrogen bonds [43]. The amide A bands were 3298.58 cm^{-1} for ASC and 3308.19 cm^{-1} for PSC, indicating the existence of hydrogen bonds. Both of amide B bands were detected at 2933.45 cm^{-1}, which was related to CH_2 asymmetrical stretching [44]. The amide I, II, III bands were associated with the carbonyl group stretching vibration coupled with a carboxyl group, N-H bending vibration coupled with a C-N stretching vibration, the combination of C-N stretching and N-H deformation from amide linkages [45], respectively. The corresponding wavelengths were measured at 1655.52 cm^{-1}, 1540.21 cm^{-1}, 1232.74 cm^{-1} for ASC and 1655.52 cm^{-1}, 1537.01 cm^{-1}, 1239.15 cm^{-1} for PSC. Moreover, the absorption ratio of amino III band and CH_2 bending band at 1450.53 cm^{-1} was close to 1.0, which proved the native and intact triple-helical structure of collagen [44, 46].

3.4. SDS-PAGE pattern

The SDS-PAGE pattern in Fig. 4 showed that ASC and PSC from Pacific cod skin were composed of two distinct α chains (α_1 and α_2) with α chain composition of $(\alpha_1)_2\alpha_2$. There were also some dimer (β chain) and trimer (γ chain) formed by intramolecular and intermolecular cross-linking of α chains. The subunit composition of ASC was identical to PSC, which indicated telopeptide did not impact on the integrity of the triple-helical structure of collagen [1]. The darker stripe of γ chain of ASC indicated the content of γ chain in ASC was notably higher than PSC, which was in accordance with the fact that ASC had more cross-linking sites in the presence of telopeptide.

3.5. Morphology of collagen sponge

In recent years, collagen sponge has been studied to be valuable in biomedical materials field. ASC had more intramolecular and intermolecular cross-linkages than PSC owing to the presence of telopeptide. These cross-linkages strengthened mechanical intensity and resistance to enzyme degradation of collagen-based biomedical materials. So ASC extracted from Pacific cod skin was used to fabricate collagen sponges for biomedical materials.

Superficial and microcosmic morphology of collagen sponge affected its biological function when applied in biomedical materials. From Fig. 5 (i), the collagen sponge was visually white, uniform, and pyknotic. And the SEM images in Fig. 5 (ii) displayed a uniform and porous microstructure. Highly porous structure is a vital character for biomedical materials. These morphology results indicated that the fabricated ASC sponge from Pacific cod skin was potential for biomedical materials.

The uniform and porous network structure of collagen sponge will play a crucial role in wound dressings, hydrating agents, matrices for cell proliferation and other biomedical materials [47].

3.6. Blood compatibility of collagen sponge

The rupture of red blood cells resulted in the release of hemoglobin into plasma and then caused the phenomenon of hemolysis. The extent of hemolysis was represented with HR value when biomedical materials contacted with blood [48,49]. In other words, few broken red blood cells indicated low HR value and good blood compatibility. Results listed in Table 2 illustrated that HR value of Pacific cod skin collagen sponge was 1.89%, lower than 5% of ASTM F 756-00, 2000. Therefore, collagen derived from Pacific cod skin was negligible hemolysis and favorable blood compatibility, which could be applied in biomedical materials field.

4. Conclusions

The extracted ASC and PSC were typical type I collagen with α chain composition of $(\alpha_1)_2\alpha_2$. In view of mechanical intensity and resistance to enzyme degradation, ASC was used to fabricate collagen sponge. The uniform and porous microstructure and good biocompatibility demonstrated the possibility of collagen sponge applied in biomedical materials field. However, further study is still needed to do to implement the application of Pacific cod skin collagen in biomedical materials field.

Declaration of interest

The authors declare no conflict of interest in this work.

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Table 1 Amino acid compositions of ASC and PSC. The results are expressed as residues/1000 total amino acid residues.

Table 2 Hemolysis ratio of Pacific cod skin collagen sponge.

Fig. 1. Thermal denaturation curve of collagen from Pacific cod skin.

Fig. 2. The curve of intrinsic viscosity of ASC and PSC.

Fig. 3. Spectroscopy properties of ASC (A) and PSC (B). (i) UV absorption spectra, (ii) X-ray diffraction spectra, and (iii) Fourier transform infrared spectra.

Fig. 4. SDS-PAGE patterns of collagen from Pacific cod skin. Lane 1: protein markers; lane 2: ASC; lane 3: PSC.

Fig. 5. Images under the stereomicroscope (I) and corresponding SEM micrographs (II) of the produced collagen sponge on surface (A) and cross section (B).

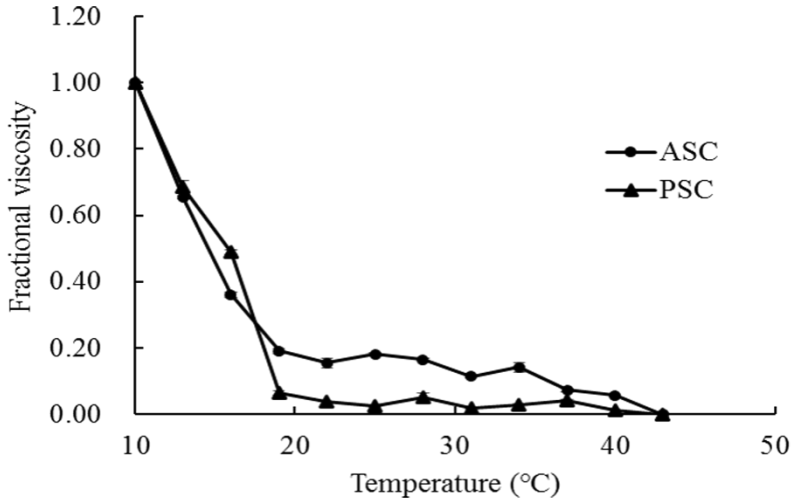
Table 1 Amino acid compositions of ASC and PSC. The results are expressed as residues/1000 total amino acid residues.

Amino acid	residues/1000 total amino acid residues	
	ASC	PSC
Aspartic acid	55	52
Threonine	23	22
Serine	61	60
Glutamic acid	81	80
Glycine	337	355
Alanine	107	108
Cysteine	2	2
Valine	21	19
Methionine	11	6
Isoleucine	13	12
Leucine	25	22
Tyrosine	4	3
Phenylalanine	14	12
Histidine	11	10
Lysine	27	27
Arginine	51	52
Proline	93	92

Hydroxyproline	64	66
Proline+ Hydroxyproline	157	159
Total	1000	1000

Table 2 Hemolysis ratio of Pacific cod skin collagen sponge.

	Hemolysis ratio (%)
Positive control	100
Negative control	0
Collagen sponge	1.89±0.44



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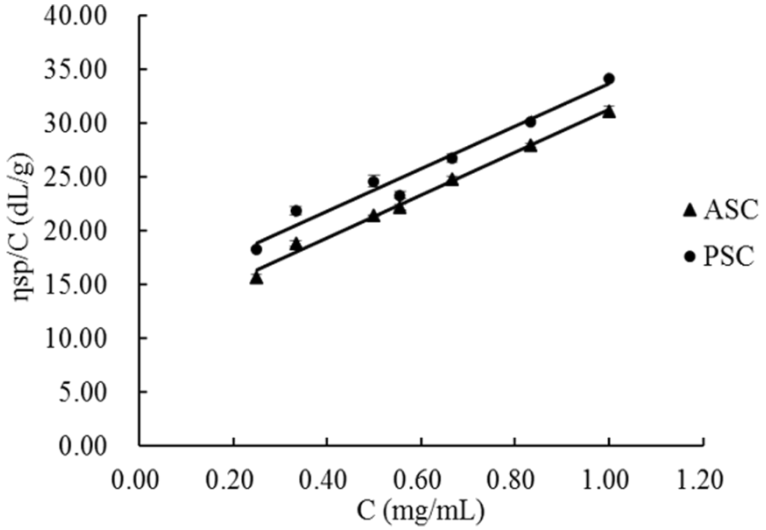
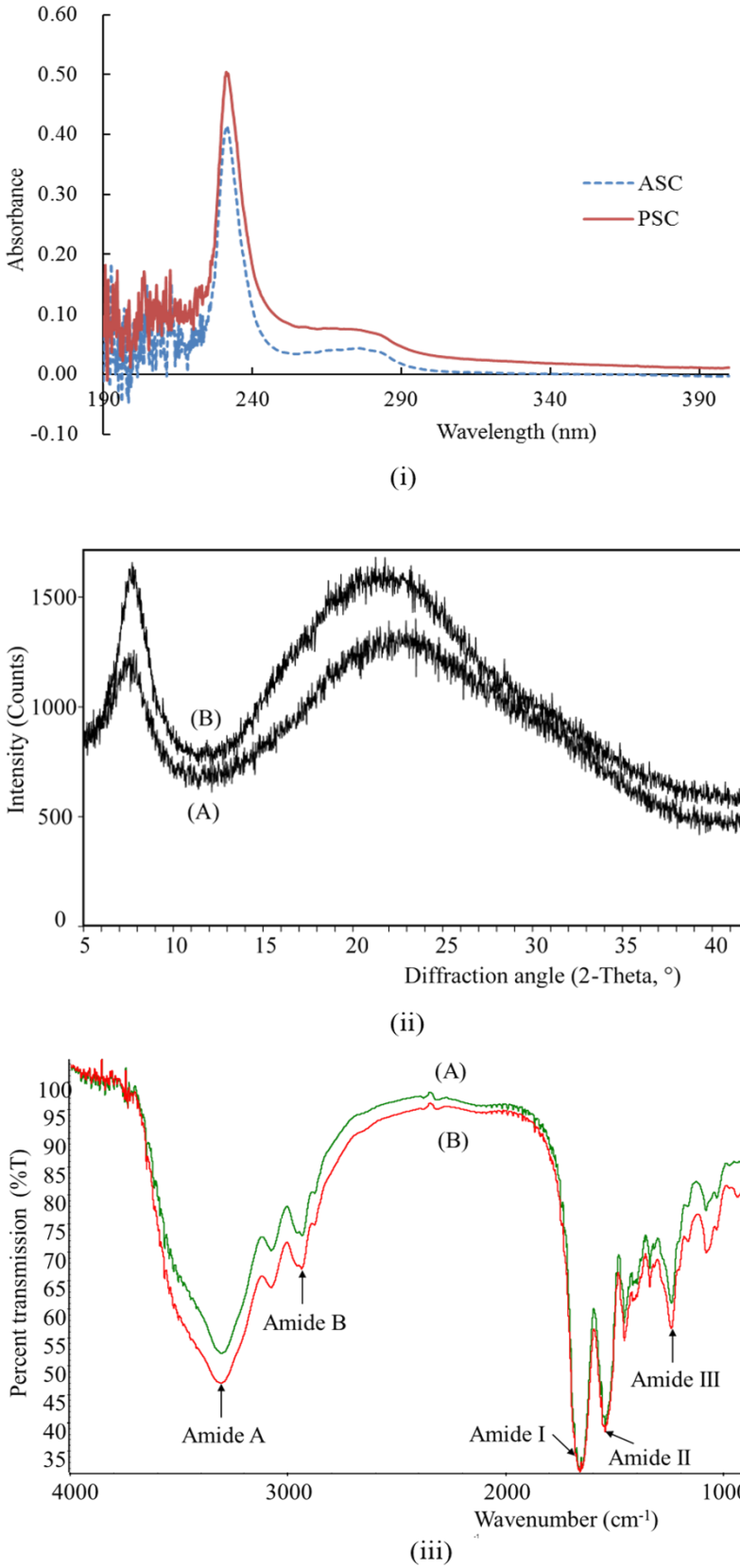


Fig. 2. The curve of intrinsic viscosity of ASC and PSC.



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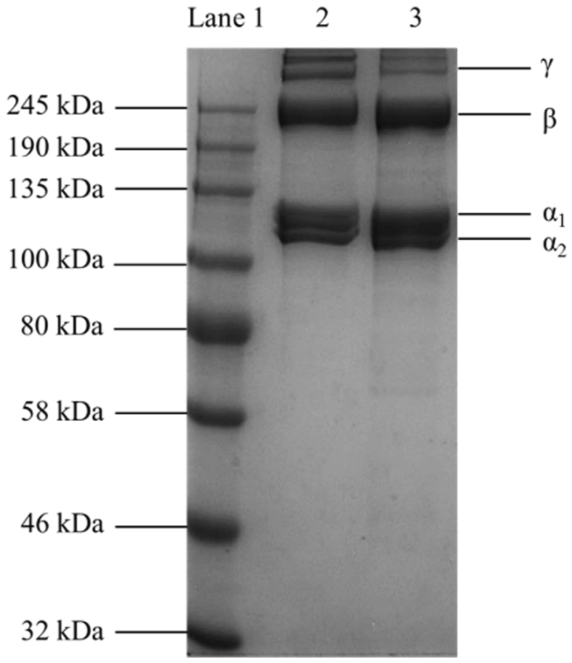

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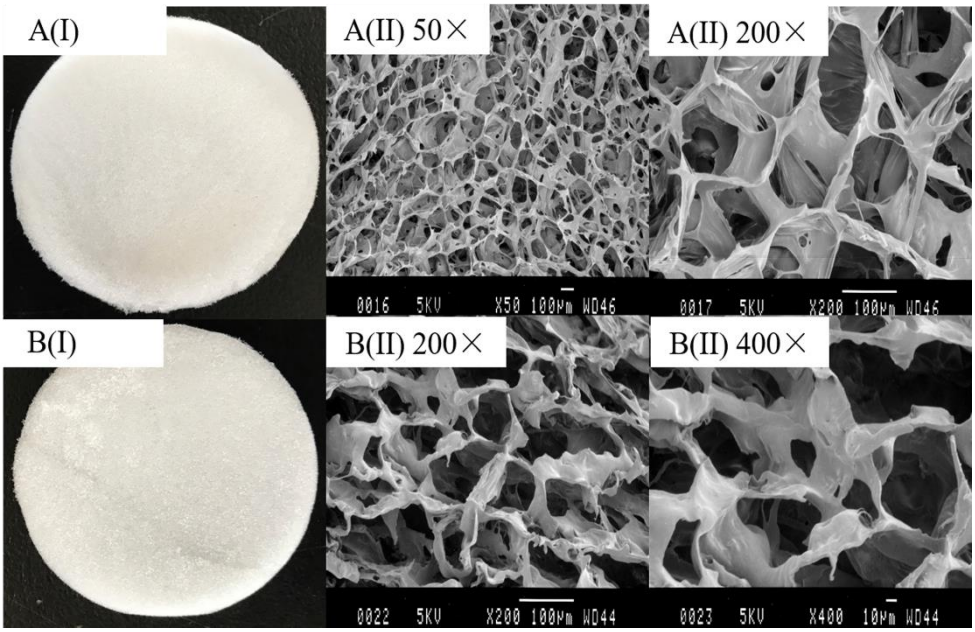

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