Biochemistry and Biophysics Reports 10 (2017) 39–45

Contents lists available at ScienceDirect

Biochemistry and Biophysics Reports

journal homepage: www.elsevier.com/locate/bbrep

Isolation and partial characterization of collagen from outer skin of Sepia pharaonis (Ehrenberg, 1831) from Puducherry coast

Jayalakshmi Krishnamoorthi[a,b], Pasiyappazham Ramasamy[a,c,*], Vairamani Shanmugam[a,d], Annaian Shanmugam[a]

[a] CAS in Marine Biology, Faculty of Marine Sciences, Annamalai University, Parangipettai 608502, Tamil Nadu, India
[b] Department of Zoology, Tagore Arts College, Puducherry 605008, India
[c] Department of Microbiology, School of Life Sciences, Pondicherry University, Puducherry 605014, India
[d] Government college of Education, Vellore, 632006 Tamil Nadu, India

ARTICLE INFO

Keywords:
Sepia pharaonis
ASC
PSC
DSC
Collagen
^{1}H-NMR

ABSTRACT

Type I collagen from outer skin of *Sepia pharaonis* was extracted and partially characterized. Yield of Acid Soluble Collagen (ASC) and Pepsin Soluble Collagen (PSC) were calculated as 1.66% and 3.93% and the total protein content of ASC and PSC were found as 18.4% and 48.6%. FT-IR spectrum of ASC and PSC recorded 12 and 14 peaks, respectively. ^{1}H NMR spectrum of ASC showed singlets at 1.23 ppm, 3.1 ppm, 3.55 ppm and 3.7 ppm and PSC at 1.23 ppm and 2.08 ppm. The molecular weight for ASC was calculated as 102 kDa and for PSC as 110, 108 and 102 kDa through SDS-PAGE. Differential Scanning Calorimetry (DSC) results supported that PSC withstand high thermal stability (82.85 °C) than ASC (73.13 °C). Higher denaturation temperature with high molecular weight well support the property of type I collagen from skin of *S. pharaonis* and it could be used as another potent source for the extraction of collagen.

1. Introduction

Collagen makes up about one fourth of the total protein content in multicellular animals [1]. Being major protein of connective tissues in animals, collagen is widely distributed in skin, bones, teeth, tendons, ligament, cartilage, eyes, blood vessels, gut, inter-vertebral discs and most other tissues and organs. Recently, alongside clarification of the biological functions of collagen as an extracellular matrix protein, it has been attracting attention as a biomaterial with many unique characteristics such as high tensile strength, low antigenicity, bioresorbability, good biocompatibility, induces coagulation of blood platelets, affects cell differentiation, wound healing, control of various characteristics through physical and chemical modifications, mouldability, abundant and easy for purification [2]. The skin of vertebrates and invertebrates are the main source of collagen used for pharmaceutical, cosmetic and biomedical research.

So far, 29 variants of collagen have been isolated that vary in the length of the helix and the nature and size of the non-helical portions [3]. The specific locations of type I collagen in animal body are bones, dermis, tendon, ligaments and cornea. Type I collagen is a glycoprotein with a carbohydrate content of less than 1%. The sugar residues are either a single galactose unit or a disaccharide of galactose and glucose O-glycosidically attached via hydrolysine residue [4]. The most abundant type of collagen is type I whose triple helix is a heteropolymer consisting of two α1 chains and one α2 chain of about 1000 residues. Stability of the triple helix in collagen depends on hydrogen bonds. Thermal denaturation of collagen depends on water content, pH of environmental medium and degree of cross-linking [5].

Collagen has been traditionally, isolated from the mammals, such as bovine and porcine and widely used in food, cosmetic, biomedical and pharmaceutical industries [6]. However, the development of Bovine Spongiform Encephalopathy (BSE) and the Foot and- Mouth Disease (FMD) crisis have resulted in anxiety among users of collagen and collagen-derived products from land-based animals in recent years [7]. Therefore, there is a strong need to develop alternative source of collagen from non-mammals [8]. Marine organisms are a rich source of structurally novel and biologically active metabolites. So far, many chemically unique compounds of marine origin with different biological activity have been isolated and a number of them are investigated and/or being developed as new pharmaceuticals [9]. The marine molluscs show extensive species diversity and their by-products have received much attention from the beginning of 20th century. Among the molluscs, some have pronounced pharmacological activities or other properties useful in the biomedical area. Furthermore, collagen

* Corresponding author at: Department of Microbiology, School of Life Sciences, Pondicherry University, Puducherry 605014, India
E-mail address: ramkanth281@gmail.com (P. Ramasamy).

http://dx.doi.org/10.1016/j.bbrep.2017.02.006
Received 20 September 2016; Received in revised form 16 February 2017; Accepted 24 February 2017
Available online 27 February 2017

has been mostly found in cephalopod skin, bone and scale [10].

In particular, cuttlefish have thick skins, but these are treated as wastes at home, fish shops, fish processing refrigerated factories. If substantial amounts of collagen could be obtained from these wastes, they would provide alternatives to mammalian collagen in foods, cosmetics and biomedical materials. It was found that good yields of collagen could be obtained from underutilized resources [10,11]. In the present investigation, an attempt has been made to explore the possibility of using the outer skin of the cuttlefish *S. pharaonis* as an additional potential source for the extraction of collagen.

2. Materials and methods

2.1. Materials

S. pharaonis were collected from Thengaithittu landing centre of Puducherry coastal region (Lat.11°54′ N; Long. 79° 49′ E), Southeast coast of India and brought to laboratory, washed immediately with tap water and then with distilled water. Finally the outer skin was carefully removed, cut into small pieces and stored at −85 °C until used.

2.2. Preparation of collagen from skin

All the preparative procedures were performed at 4 °C. The procedure of Nagai *et al.* [12] was employed for the extraction of Acid Soluble Collagen (ASC) and Pepsin Soluble Collagen (PSC) from the skin of *S. pharaonis*. Briefly the skin was extracted with 0.1 M NaOH to remove non-collagenous proteins followed by extraction with 0.5 M CH_3COOH. The supernatant was collected by centrifugation and salted out by adding NaCl. The resultant precipitate was dialyzed against 0.1 M CH_3COOH and then freeze-dried to get ASC. The residue from the CH_3COOH extraction was resuspended in 0.5 M CH_3COOH and was digested with 10% (w/v) pepsin (Sigma, USA). The pepsin-solubilized collagen was centrifuged and salted out by adding NaCl and precipitate was dialyzed against 0.1 M CH_3COOH and freeze-dried to get PSC. All the steps were carried out in less than 4 °C temperature.

2.3. Estimation of total protein

The total protein content in ASC and PSC was estimated by Lowry *et al.* [13] using bovine serum albumin (BSA) as a standard.

2.4. FT-IR spectral analysis

FT-IR spectroscopy of solid samples of standard collagen (Human placenta, Sigma, USA), ASC and PSC were relied on a Bio-Rad FT-IR–40 model, USA. Sample (10 mg) was mixed with 100 mg of dried KBr and compressed further to prepare as a salt disc (10 mm in dm) for reading the spectrum. For confirmation of extracted collagen using standard collagen as a reference apart from studying the structure.

2.5. Differential Scanning Calorimetry (DSC) of ASC and PSC

Thermogram was obtained by using Shimadzu spectrophotometer (TA instruments Q 20-DSC). 2.0 mg of lyophilized ASC sample was crimped in a standard aluminium pan and heated from 20 to 200 °C at a heating constants rate of 10° C/min under constant purging of nitrogen at 20 ml/min.

2.6. ^{1}H-NMR spectral analysis of ASC and PSC

NMR spectrum of the ASC and PSC was recorded using (BRUKER 400 MHz Ultrashield™) in DMSO solvent. The experiment was run at room temperature in which the solvent (HOD) peak does not interfere with any peaks. After dissolution, approximately 1 ml of the collagen sample solution was transferred to 5 mm NMR tube. The sample tube was inserted in the magnet and allowed to reach thermal equilibrium for 10 min before performing the experiment.

2.7. Molecular weight determination by SDS-PAGE

The gel separation (SDS -PAGE) (10%) was performed following the protocol described by Sambrook and Russell [14]. After electrophoresis, the gel was visualized with Coomassie Brilliant Blue R-250. The bands were observed under gel documentation system and the molecular weight was compared not only with the molecular marker but also with standard collagen (Sigma, USA). Finally the molecular weight of collagen was determined by using the computer package Total Lab (Version 1.11).

3. Results

3.1. Yield of collagen and protein content of ASC and PSC

In the present study, the collagen was extracted from the dried skin of *S. pharaonis* and the yield of ASC and PSC was found to be 1.66% and 3.93% on dry weight basis (DWB) and the total protein content of ASC and PSC was observed as 18.4% and 48.6% respectively.

3.2. FT-IR spectral analysis of ASC and PSC

The assignment for individual peaks of standard collagen and ASC and PSC of *S. pharaonis* skin is given in Table 1. The FT-IR spectrum of the ASC recorded 12 peaks whereas in PSC (Fig. 1B & C) reported 14 peaks which were compared with that of the standard collagen which showed 14 major peaks (Fig. 1A).

3.3. ^{1}H-NMR spectral analysis of ASC and PSC

^{1}H-NMR spectral analysis is a powerful technique to provide information about the hydrogen atom position. In the ^{1}H-NMR spectrum of ASC, the singlet peaks at 1.23 ppm, 3.1 ppm, 3.55 ppm and 3.7 ppm are due to unfolding amide and α-carbon protons and the chemical shifts of 1.23 ppm indicate the acid reacted proline and tryptophan (Fig. 2A); NMR of PSC showed two singlet peaks at 1.23 ppm and 2.08 ppm; the 2.08 ppm indicates the singlet at single proton which indicates α-carbon proton and the 1.23 ppm, the singlet at 2 proton indicates unfolding amide protons (Fig. 2B).

3.4. Differential Scanning Calorimetry (DSC) of ASC and PSC

In Differential Scanning Calorimetry (DSC), the skin ASC from *S. pharaonis* showed a “single” resolved endothermic peak in its DSC thermogram with T_o (Onset temperature) value of 44.74 °C and T_p (Peak temperature) value of 73.13 °C (Fig. 3A) and PSC also showed a

Table 1
FT–IR spectral peak and assignment for standard collagen, ASC and PSC from *S. pharaonis*.

Regions	Standard	ASC	PSC	Assignments
Amide A	3315	3448	3423	-NH- stretch coupled with hydrogen bond
Amide B	2936	2923	2923	-CH_2- asymmetrical stretch
–	2871	2853	2853	-CH_2- asymmetrical stretch
Amide I	1655	1646	1649	C=O stretch/ hydrogen bond coupled with CN stretch
Amide II	1545	1562	1557	-NH- bend coupled with CN stretch
–	1452	1464	1459	-CH_2- bend
–	1403	1413	1402	COO – symmetrical stretch
Amide III	1246	1243	1238	-NH- bend coupled with CN stretch
–	1156	–	1156	C=O stretch
–	1073	1026	1058	C=O stretch
–	619	846–610	667	Skeletal stretch

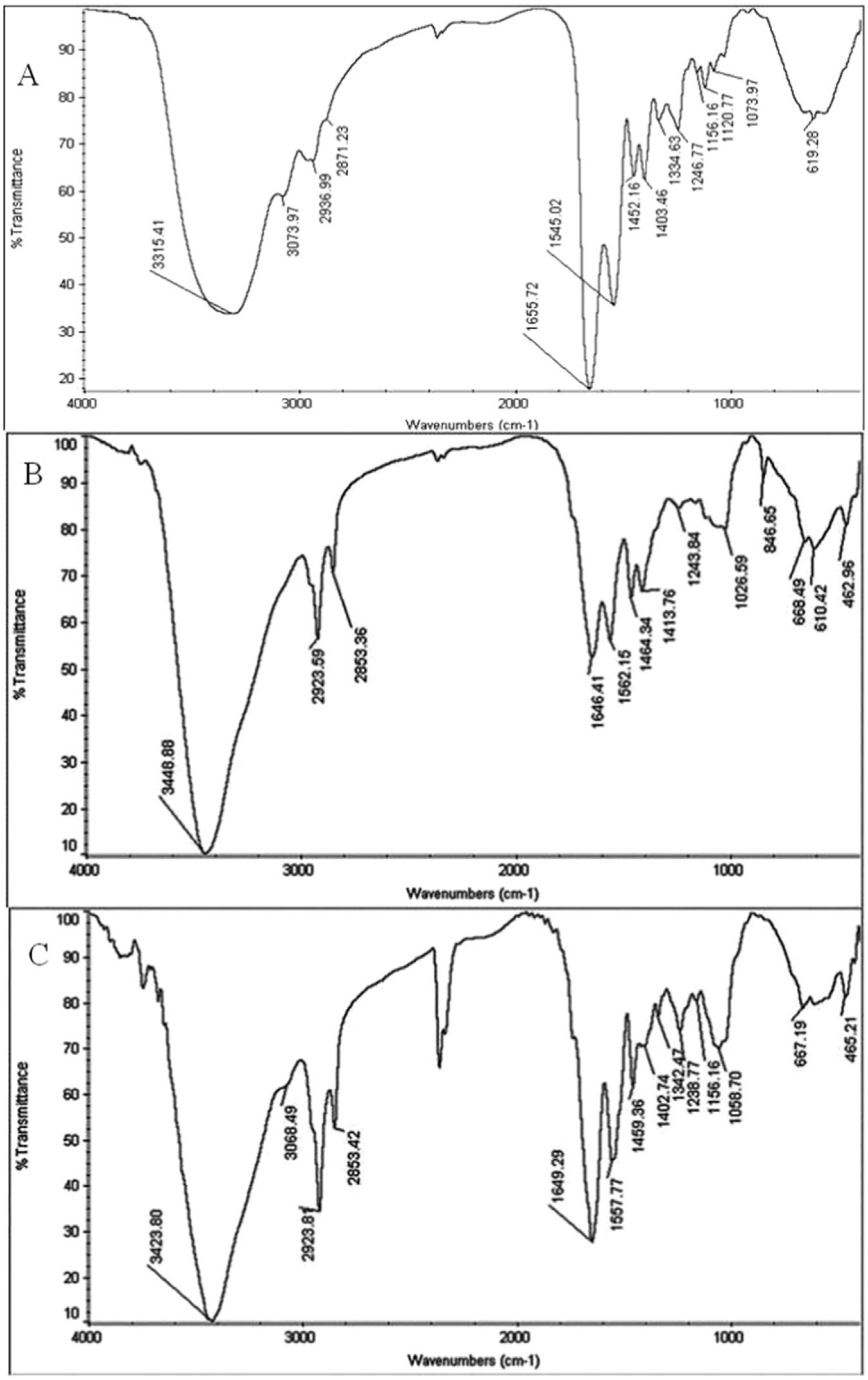

Fig. 1. FT-IR spectrum of standard collagen (A), ASC (B) and PSC (C) of *S. pharaonis*.

"single" peak with T_o value of 54.57 °C and T_p value of 82.85 °C (Fig. 3B).

3.5. Molecular weight determination by SDS-PAGE

Molecular weight of collagen is determined by SDS-PAGE. The SDS-PAGE gel profile and the pixel positions of the bands obtained for the standard collagen, protein marker and ASC & PSC from *S. pharaonis* is depicted in Fig. 4. The gel obtained through SDS - PAGE showed one band in Lane 1 representing ASC with a molecular weight of 102 kDa and in Lane 2, the standard collagen (Human placenta, Sigma, USA) was also represented by a single band with the molecular weight of 112 kDa. Whereas protein marker in Lane 3 depicted six bands with a molecular weight of 110, 97, 66, 51, 30 and 25 kDa. But the PSC in Lane 4 showed three bands with the molecular weight of 110, 108 and 102 kDa respectively (Table 2).

4. Discussion

Collagen is abundant in most of the invertebrates as well as vertebrates. It makes up about one fourth of the content in multicellular animals. The protein content of ASC and PSC, was found to be low in the present study when compared to Nagai *et al.* [12] (2% of ASC and 35% of PSC on DWB) from the skin of *Sepia lycidas*; Nagai and Suzuki [15] (ASC-5.2% and PSC-50% on DWB) from *Argonauta argo*; Sivakumar and Chandrakasan [16] 60% of PSC and 12% of ASC on wet weight basis (WWB) from the cartilage of *Sepia officinalis*; whereas, in the same animal, Sivakumar et al. [17] extracted ASC and PSC as 5.52 ± 1.3 and

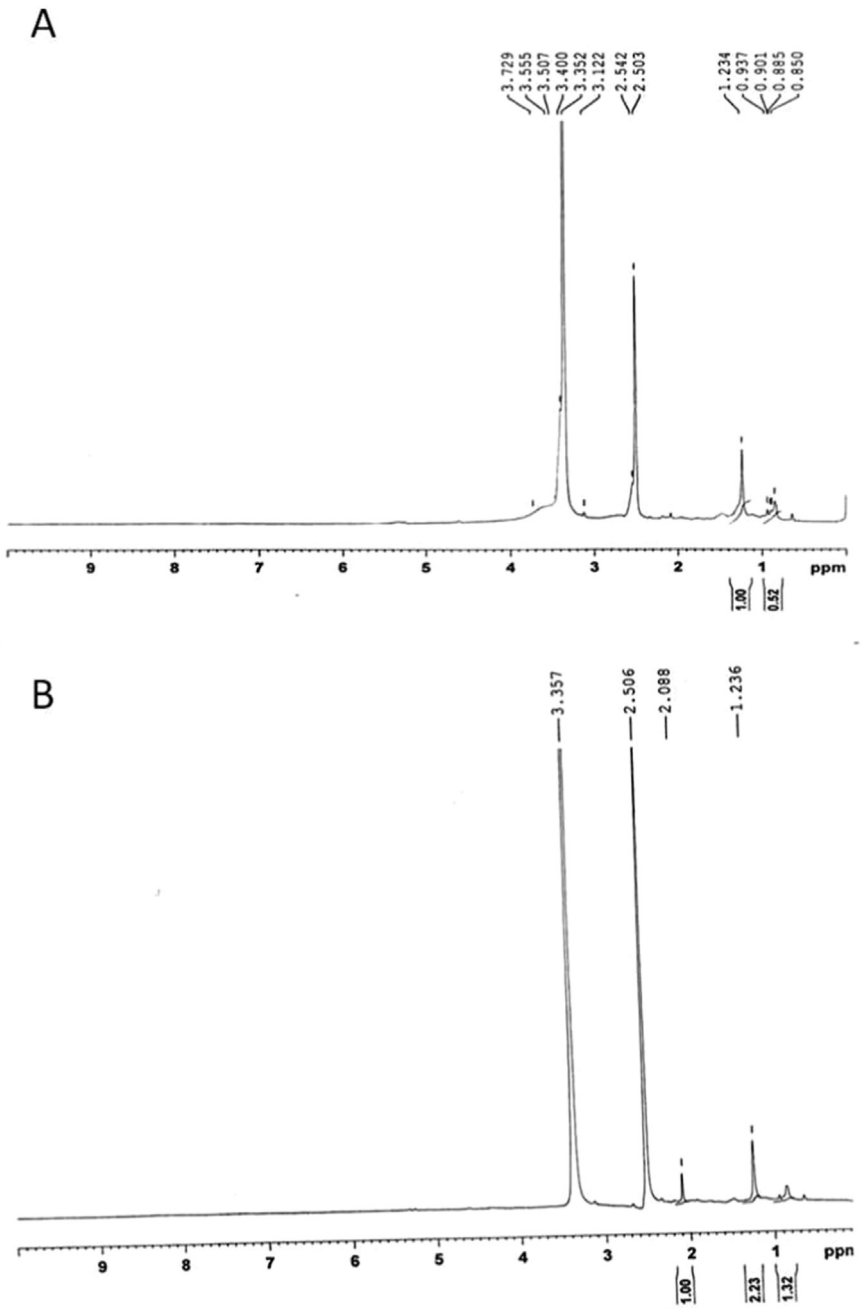

Fig. 2. NMR spectra of ASC (A) and PSC (B) of *S. pharaonis*.

27.6 ± 3.07 mg/g from the cranial cartilage and cornea, respectively; ASC and PSC from brown stripe red snapper skin as 9.0% and 4.7% (WWB) by Jongjareonrak et al. [7]. Sadowska et al. [8] reported the yield from the skin of baltic cod (*Gadus morhua*) 21.5% (WWB) and 71.2% (DWB); whereas Zeng et al. [18] extracted ASC from the skin of *Octopus niloticus* as 39.4% on DWB. When compared to the present study, lesser collagen content in arm and mantle of *O. vulgaris*, (1.4% and 1.9% on WWB) in arm and mantle, respectively and lesser protein content (9.1% and 14%, respectively) by Mizuta et al. [19]; lesser ASC (1.30%) from *Thysanoteuthis rhombus,* higher PSC (35.6%) on DWB [20]. Whereas in *Sepiella inermis* [21] and *Sepioteuthis lessoniana* [22] reported the yield and protein in ASC and PSC were 0.58%, 16.23% and 20.24%, 69.56% and 3.83%, 11.25% and 79.7%, 87.75% on DWB respectively.

In generally the PSC have greater yield collagen, as compared to the ASC. The ASC results might be attributed to the low solubility of crosslinks formed via the reaction of aldehyde with lysine and hydroxylysine at telopeptide helical sites. With further limited pepsin digestion, the cross-linked molecules at the telopeptide region were most likely cleaved, resulting in the increased collagen extraction efficacy. Pepsin was able to cleave specifically at the telopeptide region of collagen from snakehead fish scale [23]. This might be the main reason the PSC method produces a higher amount of collagen. This variation in the amount of collagen may be due to the concentration of acetic acid used and reduced solubility of collagen in the extraction solvent. Further the low yield of collagen content may be due to the denaturation of protein during the process of methodology and difference in environmental temperature [24]. The high yield of total protein in *S. pharaonis* may be due to the reason that the skin contains more protein when compared to arm and mantle as observed by Takema and Kimura [25] in the case of the minor collagen type in the octopus arm muscle.

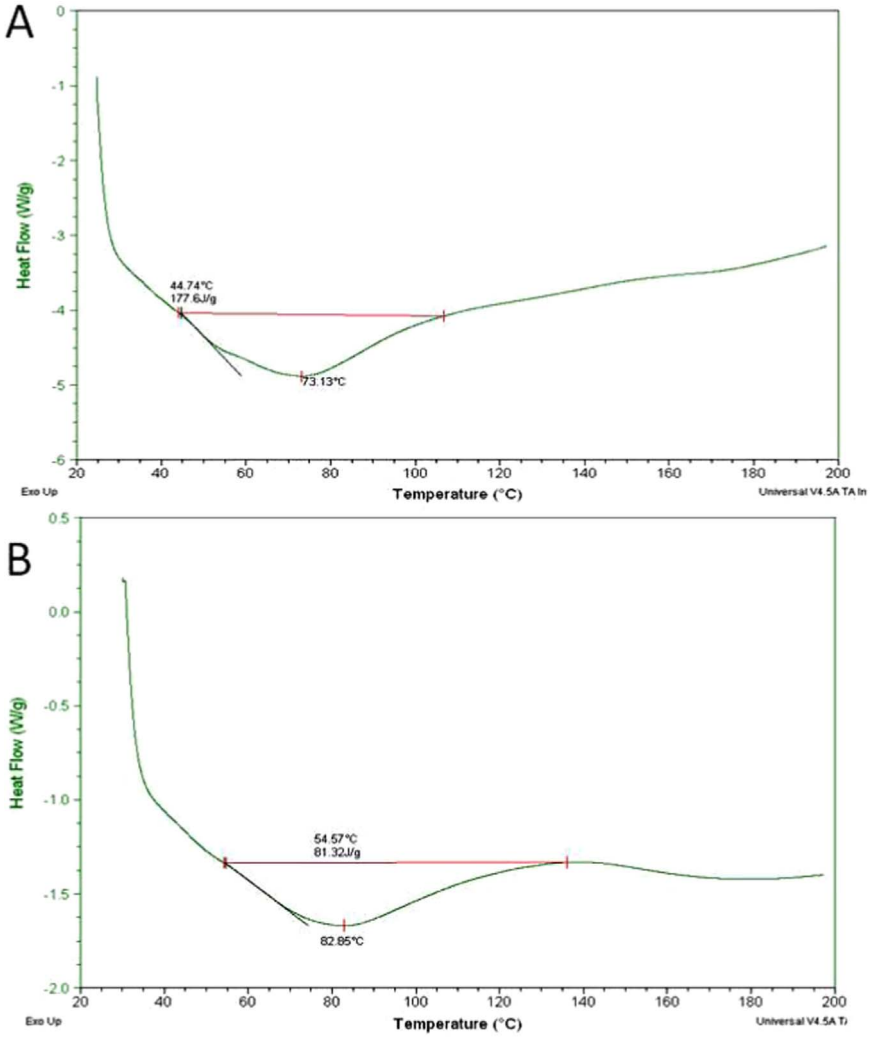

Fig. 3. DSC thermogram ASC and PSC of *S. pharaonis*.

The regions of amides I, II and III are known to be directly related with the shape of a polypeptide. Amide A band (3400–3440 cm^{-1}) is related to N-H stretching vibrations. Amide I band (1600–1660 cm^{-1}) is associated with stretching vibrations of carbonyl groups in peptides, being the most important factor in investigating the secondary structure of protein. Amide II (~1500 cm^{-1}) is associated with NH bending and CN stretching. Amide III (1320–1220 cm^{-1}) is related to CN stretching and NH bending and it is involved with the triple helix structure of collagen [26–28]. The FT-IR spectrum of *S. pharaonis* for ASC, the band started at 3448 cm^{-1} and down to 610 cm^{-1}. The amide region bands of A, B, I, II and III are at 3448 cm^{-1}, 2923 cm^{-1}, 1646 cm^{-1}, 1562 cm^{-1} and 1243 cm^{-1}; for PSC, the band started at 3423 cm^{-1} and down to 667 cm^{-1}. The amide region bands of A, B, I, II and III are at 3423 cm^{-1}, 2923 cm^{-1}, 1649 cm^{-1}, 1557 cm^{-1} and 1238 cm^{-1}.

Wang et al. [29] reported that amide A band of skin collagen of *Sebastes mentella* was at 3425 cm^{-1}, while those of scale and bone were at 3296 and 3300 cm^{-1}, respectively, indicating more NH groups of scale and bone were involved in hydrogen bonding than in skin. Comparing to this, in the present investigation, the amide A band of ASC and PSC were located at 3448 and 3423 cm^{-1}, respectively, also indicating the involvement of more NH groups in hydrogen bonding of PSC than ASC. The Amide B band of both collagens was observed from 2921 to 2925 cm^{-1}, in agreement with previous reports [12].

The absorption between the 1241 and 1240 cm^{-1} (amide III) and 1458 cm^{-1} (ASC and PSC respectively) wavelength demonstrated the existence of helical structure [30]. 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 [31]. Due to the similarity in the amplitude, both collagens were most likely not denatured during the extraction. This was reconfirmed by the ratio of ~1 between amide III and 1454 cm^{-1} band of both collagens. Ratio of ~1 revealed the triple-helical structure of collagen. The amide II of both collagens appeared at 1538–1541 cm^{-1}, resulting from N–H bending vibration coupled with CN stretching vibration [32]. Thus, both ASC and PSC showed a similar secondary structure.

The peak band of amide I, with characteristic frequencies between 1600 cm^{-1} and 1700 cm^{-1} is mainly associated with the stretching vibrations of carbonyl groups (C=O bond) along the polypeptide [33] and was a sensitive marker of peptide secondary structure [27]. In the present investigation on *S. pharaonis* skin collagen, the amide I band position was observed at 1646 cm^{-1} in ASC and 1649 cm^{-1} in PSC, which is the absorption band of C=O stretching and is responsible for secondary structure of peptide. Similarly transmission peaks of amide III at 1243 cm^{-1} and 1459 cm^{-1} (ASC and PSC) confirms triple helical structure of collagen from the skin of *S. pharaonis* [30]. Amide B band of both collagens was observed at 2923 − 2923 cm^{-1}, which is in agreement with that reported by Nagai et al. [12]. The amide A band of skin collagen of *S. mentella* was at 3425 cm^{-1}, while those of scale and bone were at 3296 cm^{-1} and 3300 cm^{-1} respectively, which indicate that more NH groups of scale and bone were involved in hydrogen bonding than in skin [29]. Comparing to this, in the present study also the amide

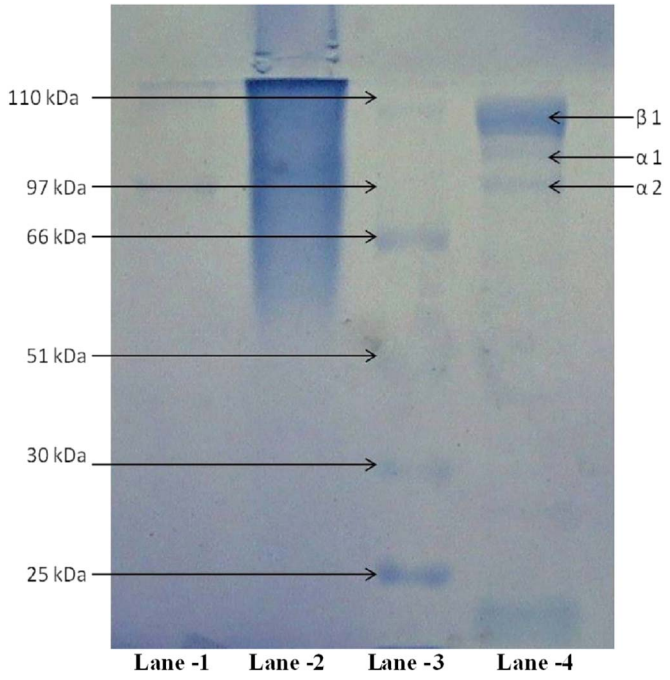

Fig. 4. SDS-PAGE electrophoresis pattern of *S. pharaonic* (Lane 1-ASC; Lane 2-Std. collagen; Lane 3- Protein marker; Lane 4- PSC).

A band of ASC and PSC are located at 3448 and 3423 cm^{-1} respectively; it also indicates the involvement of more number of NH groups in hydrogen bonding of ASC and PSC. C = O bond along the polypeptide backbone [33] was a sensitive marker of the peptide secondary structure [27].

The chemical shift of the proline and tryptophan found at 1.9 ppm and 3.3 ppm [34] respectively coincided with those found in the ASC and PSC of *S. pharaonis* collagen also. The presence of the triple-helical conformation in the peptide solution can also be established by ^{1}H NMR spectroscopy. The assembly of a triple-helical structure results in the appearance of a new set of NMR resonances, which cannot be observed for the unassembled structures [35–37]. Among the resonances of the assembled triple-helical set, the signal of proline at 3.1 ppm is well resolved and not overlapped by any resonance of the unfolded structure sets. The resonance at 3.1 ppm can therefore be used unambiguously to identify the triple-helical structure [38]. In the present study a strong peak signal at near 3.1 ppm is seen in ASC, which confirm the triple-helical structure of collagen.

In DSC it may be noticed, that the PSC values are higher than that of the ASC values which suggest that PSC possesses more cross-linking linkages than ASC. The high enthalpy of unfolding of collagen is thought to derive mainly from the breaking of the hydrogen bonds between triple helixes and hydrogen bonds forming the hydration network around the collagen molecule. The decrease of the enthalpy of this degenerated structure is attributed to the loss of bound water and thermal cooperation of the components.

Thermal stability of collagen is usually described by the denaturation temperature (*Td*). The temperature at which the triple helix structure of collagen is disintegrated into random coils is considered as *Td* [38]. In the present study of thermo-physical properties of the collagen by DSC, the peak temperature of the collagen was reported to be 75.93 °C for ASC and 75.05 °C for PSC; but at the same time the peak temperature of the collagen membrane was about 59 °C [39]. Td of collagens from skins of different fishes ocellate puffer fish (28.0 °C) [15], grass carp (28.4 °C) [40], brown backed toadfish (28.0 °C) [41], cod (15.0 °C) [42]. whereas the DSC results of the present study for the *S. pharaonis* skin collagen are very high than the above said results; the difference may be due to the thermal behavior of collagen, which is related to the moisture content, the thermal history (that is, preparative conditions), type/species of fish and season and region of capture. Generally, collagens obtained from fish species living in cold environments have lower contents of hydroxyproline and they exhibit lower thermal stability than those from fish living in warm environments [28]. Varying thermal transition or denaturation temperatures depend on fish species, habitat temperature, where the fish live, seasons and age [43]. Further in the presence of 0. 05 M acetic acid, the peak was shifted to lower temperature. Thus acetic acid might change the confirmation of collagens in the fashion which was more prone to denaturation. The inter-chain hydrogen bonds stabilizing the collagen triple-structure where partially cleaved by acetic acid. On the other hand, the stability of collagen is also known to be correlated with the environmental and body temperatures [42]. Thus, collagen from *S. pharaonis* skin had the high thermal stability, which might be associated with the different properties of collagen from the species.

In the present study SDS-PAGE recorded the presence of one (α1) and three (α1, α2 and β) chains in ASC and PSC respectively. In addition, β-chain, representing dimer, was observed only in PSC. Nevertheless the band intensity of PSC β-chain was higher than that of ASC. The result indicated that PSC contained higher proportion of inter- and intra-crosslink like β (dimers) than did ASC. The inter-molecular and intra-molecular cross linked components found in *S. pharaonis* skin collagen were found similar to those of bigeye snapper [44] and ocellate puffer fish skin [45]. Existence of at least two different subunits (α1and α2) showed that major collagen from Walley Pollock skin might be the type I collagen [46]. This is accordant with the present study, where the molecular weight was observed between 102 and 110 kDa which is similar to that of type I collagen.

5. Conclusions

Cuttlefishes have thick skin, but this (skin) is treated as waste at home, in the fish shops, processing units, seafood industries and refrigerated factories. If substantial amounts of collagen could be obtained from this waste, it would provide alternatives to mammalian collagen in food, cosmetics and biomedical materials. Hence, the collagen (ASC and PSC) was extracted from the outer skin of cuttlefish *S. pharaonis* and characterized. Based on electrophoretic mobility pattern, FT-IR and DSC results, it can be concluded that collagen isolated from *S. pharaonis*, is the type I collagen since it has higher denaturation temperature with the molecular weight lying between 102 and 110 kDa which is similar to that of type I collagen and well support

Table 2
Molecular weight (in kDa) of ASC and PSC from *S. pharaonis*.

Lane 1 (ASC)			Lane 2 (Std. collagen)			Lane 3 (Protein marker)			Lane 4 (PSC)		
Band	Vol.	MW	Band	Vol.	MW	Band	Vol.	MW	Band	Vol.	MW
1	213,065	102	1	224,303	112	1	180,600	110	1	213,094	110
						2	208,083	97	2	113,750	108
						3	251,810	66	3	120,395	102
						4	173,156	51			
						5	212,806	30			
						6	320,723	25			

the property of type I collagen in ASC and PSC of *S. pharaonis* skin. From the results, it may be inferred that skin waste of cuttlefish from the processing plants as an alternative source for the present day conventional source of collagen for industrial purposes.

Conflict of interest statement

We declare that we have no conflict of interest.

Acknowledgement

Authors are thankful to the Director and Dean, Centre of Advanced Study in Marine Biology, Faculty of Marine Sciences, Annamalai University for providing the necessary facilities.

References

[1] A. Bailey, M. Floorkin, E.H. Stole (Eds.), The nature of collagen, Comprehensive Biochem, 1968, pp. 591–599.
[2] K.H. Stenzel, T. Miyata, A.L. Rubin, Collagen as a biomaterial, Annu. Rev. Biophys. Bioeng. 3 (1974) 231–253.
[3] C. Shoderhall, I. Marenholz, T. Kerscher, F. Ruschendorf, J. Esparza-Gordillo, M. Worm Variants in a novel epidermal collagen gene (COL29A1) are associated with atopic dermatitis. PLoS *Bio* 200; 5: 19-52.
[4] Piez K.A. Collagen, J.I. Kroschwitz, Ency Poly Sci Eng, Wiley, New York, 1985, pp. 699–727.
[5] R. Usha, T. Ramasami, The effects of urea and n-propanol on collagen denaturation: using DSC, circular dicroism and viscosity, Thermochim. Acta 409 (2004) 201–206.
[6] M. Ogawa, R.J. Portier, M.W. Moody, J. Bell, M.A. Schexnayder, J.N. Losso, Biochemical properties of bone and scale collagens isolated from the subtropical fish black drum (*Pogonia cromis*) and sheep head sea bream (*Archosargus probatocephalus*), Food Chem. 88 (2004) 495–501.
[7] A. Jongjareonrak, S. Benjakul, W. Visessanguan, T. Nagai, M. Tanaka, Isolation and characterization of acid and pepsin - solubilized collagens from the skin of brown stripe red snapper (*Lutjianus vita*), Food Chem. 93 (2005) 475–484.
[8] M. Sadowska, I. Kolodziejska, C. Niecikowska, Isolation of collagen from the skin of Baltic cod (*Gadus morhua*), Food Chem. 81 (2003) 257–262.
[9] R. Ely, S. Supriya, C.G. Naik, Antimicrobial activity of marine organisms collected off the coast of Southeast India, J. Exp. Mar. Biol. Ecol. 309 (2004) 121–127.
[10] T. Nagai, N. Suzuki, Isolation of collagen from fish waste materials – skin, bone and fins, Food Chem. 68 (2000) 277–281.
[11] T. Nagai, W. Worawattanamateekulm, N. Suzukim, T. Nakamuram, T. Itom, K. Fujikim, M. Nakao, T. Yano, Isolation and characterization of collagen from Rhizostomous jellyfish (*Rhopilema asamushi*), Food Chem. 70 (2000) 205–208.
[12] T. Nagai, E. Yamashita, K. Taniguchi, N. Kanamori, N. Suzuki, Isolation and characterization of collagen from the outer skin waste material of cuttlefish (*Sepia lycidas*), Food Chem. 72 (2001) 425–429.
[13] O.H. Lowry, N.J. Rosebrough, A.L. Farr, R.J. Randall, Protein measurement with the folin-phenol reagent, J. Biol. Chem. 193 (1951) 265–275.
[14] J. Sambrook, D.W. Russell, Molecular cloning laboratory manuals, cold spring harbor, 3rd edition, Cold spring harbor laboratory press, New York, 2001, pp. 40–49.
[15] T. Nagai, N. Suzuki, Preparation and partial characterization of collagen from paper nautilus (*Argonauta argo*, Linnaeus) outer skin, Food Chem. 76 (2002) 149–153.
[16] P. Sivakumar, G. Chandrakasan, Occurrence of a novel collagen with three distinct chains in the cranial cartilage of the squid *Sepia officinalis*: comparison with shark cartilage collagen, Biochim. Biophys. Acta 1381 (1998) 161–169.
[17] P. Sivakumar, L. Suguna, G. Chandrakasan, Similarity between the major collagens of cuttlefish cranial cartilage and cornea, Comp. Biochem. Physiol. 134B (2003) 171–180.
[18] S. Zeng, C. Zhang, H. Lin, P. Yang, P. Hong, Z. Jiang, Isolation and characterization of acid–solubilised collagen from the skin of Nile tilapia (*Oreochromis niloticus*), Food Chem. 116 (2009) 879–883.
[19] S. Mizuta, T. Takahidae, R. Yoshinaka, Comparison of collagen types of arm and mantle muscles of the common Octopus (*Octopus vulgaris*), Food Chem. 81 (2003) 527–532.
[20] T. Nagai, Collagen from diamondback squid (*Thysanoteuthis rhombus*) outer skin, Z. Nat. C. Biol. Sci. Tub. 59c (2004) 271–275.
[21] S. Vairamani, P. Ramasamy, N. Subhapradha, S. Sudharsan, P. Seedevi, M. Moovendhan, K. Jayalakshmi, A. Shanmugam, A. Srinivasan, Extraction, structural and physical characterization of type I collagen from the outer skin of *Sepiella inermis*, Afr. J. Biotechnol. 11 (2012) 14326–14337.
[22] P. Ramasamy, N. Subhapradha, S. Vairamani, A. Shanmugam, Isolation and structural characterisation of acid- and pepsin soluble collagen from the skin of squid *Sepioteuthis lessoniana* (Lesson, 1830), Nat. Prod. Res. 28 (2014) 838–842.
[23] H.S. Jeong, J. Venkatesan, S.K. Kim, Isolation and characterization of collagen from marine fish (*Thunnus obesus*), Biotechnol. Bioprocess Eng. 18 (2013) 1185–1191.
[24] C. Rigo, D.J. Hartmann, A. Bairan, Eletrophotertic study of collagens from *Sepia officinalis* cartilage, Biochim. Biophys. Acta 1572 (2002) 77–84.
[25] Y. Takema, S. Kimura, Two genetically distinct molecular species of Octopus muscle collagen, Biochim. Biophys. Acta 706 (1982) 123–128.
[26] A. Jakobsen, P.B. Kristensen, H.K. Poulsen, Flow cytometric classification of biopsy specimens from cervical intraepithelial neoplasia, Cytomet 41 (1983) 66–171.
[27] W.K. Surewicz, H.H. Mantsh, New insight into protein secondary structure from resolution enhanced infrared spectra, Biochim. Biophys. Acta 952 (1988) 115–130.
[28] J.H. Muyonga, C.G.B. Cole, K.G. Duodu, Characterization of acidic soluble collagen from skins of young and adult Nile Perch (*Lates niloticus*), Food Chem. 85 (2004) 81–89.
[29] L. Wang, X. An, Z. Xin, L. Zhao, Q. Hua, Isolation and characterization of collagen from the Skin, scale and bone of deep-sea red fish (*Sebastes mentella*), Food Chem. 108 (2008) 616–623.
[30] H.Y. Liu, D. Li, S.D. Guo, Studies on collagen from the skin of channel catfish (*Ictalurus punctatus*), Food Chem. 101 (2007) 621–625.
[31] M.A. Bryan, J.W. Brauner, G. Anderle, C.R. Flach, B. Brodsky, R. Mendelsohn, FT-IR studies of collagen model peptides: complementary experimental and simulation approaches to conformation and unfolding, J. Am. Chem. Soc. 129 (2007) 7877–7884.
[32] S. Krimm, J. Bandekar, Vibrational spectroscopy and conformation of peptides, polypeptides and proteins, Adv. Protein Chem. 38 (1986) 181–364.
[33] K.J. Payne, A. Veis, Fourier transform infra-red spectroscopy of collagen and gelatin solutions: deconvolution of the Amide I band for conformational studies, Biopoly 27 (1988) 1749–1760.
[34] A. Bundi, K. Wuethrich, Proton NMR parameters of the common amino acid residues measured in aqueous solutions of the linear tetra peptides H-Gly-Gly-X-L-Ala-OH, Biopoly 18 (1979) 285–297.
[35] B. Brodsky, M.H. Li, C.G. Long, J. Apigo, J. Baum, NMR and C + D studies of triple-helical peptides, Biopoly 32 (1992) 447–451.
[36] M. Li, P. Fan, B. Brodsky, J. Baum, Two-dimensional NMR assignments and conformation of (Pro-Hyp-Gly) 10 and a designed collagen triple-helical peptide, Biochem 32 (1993) 7377–7387.
[37] G. Melacini, Y. Feng, M. Goodman, Acetyl-terminated and template-assembled collagen-based polypeptides composed of Gly-Pro-Hyp sequences. 3. Conformational analysis by ^{1}H-NMR and molecular modeling studies, J. Am. Chem. Soc. 118 (1996) 10359–10364.
[38] L.H. Hao, B.F. Li, Extraction of collagen from body wall of *Asterias amurensis*, J. Fish. Sci. Chin. 6 (1999) 18–21.
[39] Z. Zhang, G. Li, B. Shi, Physicochemical properties of collagen, gelatin and collagen hydrolysate derived from bovine limed split wastes, J. Soc. Leath Tech. Che. 90 (2005) 23–30.
[40] Y. Zhang, W. Liu, G. Li, B. Shi, Y. Miao, X. Wu, Isolation and partial characterization of pepsin-soluble collagen from the skin of grass carp *Ctenopharyngodon idella*, Food Chem. 103 (2007) 906–912.
[41] L.S. Senaratne, P. Park, S. Kim, Isolation and characterization of collagen from brown backed toadfish (*Lagocephalus gloveri*) skin, Biores. Technol. 97 (2006) 191–197.
[42] B.J. Rigby, Amino-acid composition and thermal stability of the skin collagen of the Antarctic ice-fish, Nature 219 (1968) 166–167.
[43] R. Duan, J. Zhang, X. Du, X. Yao, K. Konno, Properties of collagen from skin, scale and bone of carp (*Cyprinus carpio*), Food Chem. 112 (2009) 702–706.
[44] P. Kittiphattanabawon, S. Benjakul, W. Visessanguan, H. Kishimura, F. Shahidi, Isolation and Characterization of collagen from the skin of brown banded bamboo shark (*Chiloscyllium punctatum*), Food Chem. 119 (2010) 1519–1526.
[45] T. Nagai, Y. Araki, N. Suzuki, Collagen of the skin of ocellate puffer fish (*Takifugu rubripes*), Food Chem. 781 (2002) 73–177.
[46] M. Yan, B. Li, X. Zhao, G. Ren, Y. Zhuang, H. Hou, X. Zhang, L. Chen, Y. Fan, Characterization of acid-soluble collagen from the skin of walleye pollock (*Theragra chalcogramma*), Food Chem. 107 (2008) 1581–1586.