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RESEARCH ARTICLE

ISOLATION AND CHARACTERIZATION OF COLLAGEN FROM THE SKIN OF *Sepia pharaonis* (Ehrenberg, 1831)

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ABSTRACT

In the present study an attempt has been made to isolate and characterize the type I collagen from outer skin of *Sepia pharaonis*. The total protein content and molecular weight of acid soluble collagen (ASC) and pepsin soluble collagen (PSC) were determined. The structure of ASC and PSC from *S. pharaonis* was persistent by using Fourier transform infrared spectroscopy (FT-IR) and UV *vis* spectrum. The soluble collagen was extracted by treating the skin with 0.5 M CH_3COOH and centrifuged (ASC). Then residue was resuspended in 0.5 M CH_3COOH and was digested with 10% (w/v) pepsin (PSC). On the basis of dry weight the ASC and PSC content was observed as 1.70% and 3.61% and the total protein content of both ASC and PSC was found to be 16.4% and 44.6% respectively. The molecular weight of ASC and PSC was calculated as 107 kDa and 73 to 117 kDa respectively. Both ASC and PSC consisted of two different α chains (α 1 and α 2), and were characterized to be type I with no disulfide bond. PSC had a higher content with high molecular weight cross-links, than did ASC. The results of this study suggest that the skin of *S. pharaonis* could be used as another potent source for the exploration of collagen.

INTRODUCTION

Collagen is an abundant protein in vertebrates and constitutes 30% of total animal protein (Muyonga *et al.,* 2004). Collagen has been traditionally, isolated from the mammals, such as bovine and porcine, and widely used in food, cosmetic, biomedical and pharmaceutical industries (Ogawa *et al.,* 2004). 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 (Jongjareonrak *et al.,* 2005). Additionally, collagen obtained from pig cannot be used as a component of some food for religious reasons moreover it is not easy to distinguish between the two sources. Therefore, there is a strong need to develop alternative source of collagen from non-mammals (Sadowska *et al.,* 2003). 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 (Ely *et al.,* 2004). 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. It is surprising that some of these pharmacological activities are attributed to the presence of polysaccharides, particularly those that are sulfated (Arumugam and Shanmugam, 2004).Furthermore, collagen has been mostly found in cephalopod skin, bone and scale (Kimura, 1992; Nomura *et al.,* 1996; Nagai and Suzuki, 2000a; Ikoma *et al.,* 2003). So far, nineteen variants of collagen have been reported, named type I–XIX (Bailey et al., 1998). Mostly the skins are having the type I collagen. In the present investigation, an attempt has been made to explore the possibility of using the outer skin of the cuttlefish Sepia pharaonis as a source for the extraction of collagen.

During cephalopod processing, a large amount of wastes is generated. Cephalopod skin wastes contribute 50–70% of the original raw material, depending on the processes used and types of products. Those wastes have been of interest as high-protein human foods, instead of employing them as pet foods (Montero *et al.,* 1991; Shahidi, 1994). Among the value-added products derived from those wastes, collagen from skin, scale and bone has increasingly been of interest owing to its abundance. Therefore a great amount of food is dumped as waste and a lot of leftover food is dumped as domestic waste. Although there is a drift to decrease the waste in the world, the

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quantity produced is increasing year by year. Recently, there has been a lot of interest in investigating possible means of making more effective use of under-utilized resources and industrial wastes. In particular, cuttlefish have thick skins, but these are treated as wastes at home, fish shops, fish processing and refrigerated factories. If these wastes were dumped as it is, they would cause pollution and offensive odour. If substantial amounts of collagen could be obtained from these wastes, they would provide alternatives to mammalian collagen in foods, cosmetics and biomedical materials. During studies on underutilized resources, it was found that good yields of collagen could be obtained from some underutilized resources (Nagai and Suzuki, 2000a, b, c; Nagai *et al.,* 1999, 2000). The present investigation also aims to prove the possibility of using the skin of *S. pharaonis* as an additional potential source of collagen.

MATERIALS AND METHODS

Sampling

The samples were collected from Cuddalore landing centre (Lat.11°42' N; Long. 79°46'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 and stored at -80°C until use.

Preparation of collagen from skin

The procedure of Nagai *et al.* (2001) was employed for the extraction of ASC and 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 re-suspended 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 4ºC temperature.

Total protein

Total protein content of ASC and PSC was estimated by following the standard method of Lowry *et al.* (1951).

FT-IR spectral analysis

FT-IR spectroscopy of solid samples of standard, 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.

UV-vis spectra

The collagen sample was dissolves in 0.5 M acetic acid and UV- *vis* adsorption spectra were recorded according to the method of Yan *et al.* (2008). The UV-*vis* adsorption spectra were recorded in a Shimadzu spectrophotometer UV -1800.

SDS-PAGE

The SDS-PAGE (10%) electrophoresis was performed by following the protocol described by Sambrook and Russell (2001). After electrophoresis, the gel was visualized with Coomassie Brilliant Blue R-250. The bands were observed under gel documentation system and molecular weight was determined using molecular marker (Sigma, USA).

RESULTS

Yield of collagen

The yield of ASC and PSC was found to be 1.70% and 3.61% on the basis of dry weight of the skin of *S. pharaonis* respectively.

Total protein content

The total protein content of ASC and PSC in the skin of *S pharaonis* was observed as 16.4% and 44.6% respectively.

FT – IR spectral analysis

The FT-IR spectrum of the ASC (Fig. 1) recorded 12 peaks whereas in PSC (Fig. 2) reported 9 peaks which were compared with that of the standard (Fig. 3) which showed 17 major peaks. The assignment for individual peaks of standard collagen and ASC and PSC of *S. pharaonis* skin is given in Table 1.

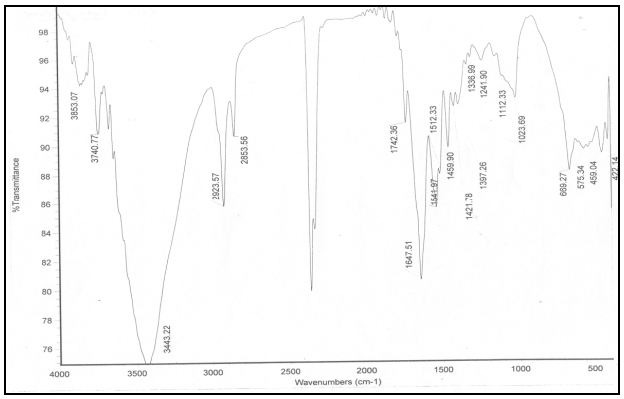


Fig. 1. Showing the FT-IR spectrum of ASC of S. pharaonis

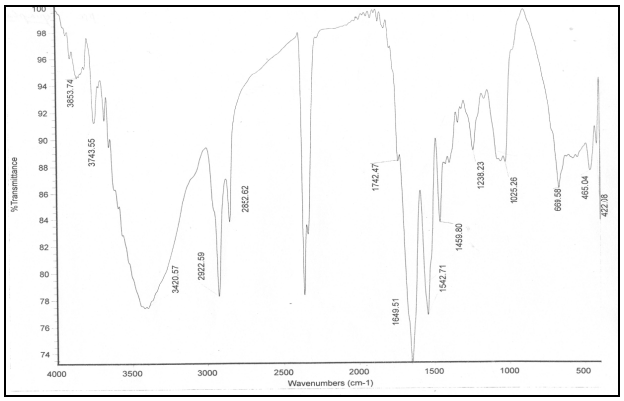


Fig. 2. Showing the FT-IR spectrum of PSC of *S. pharaonis*

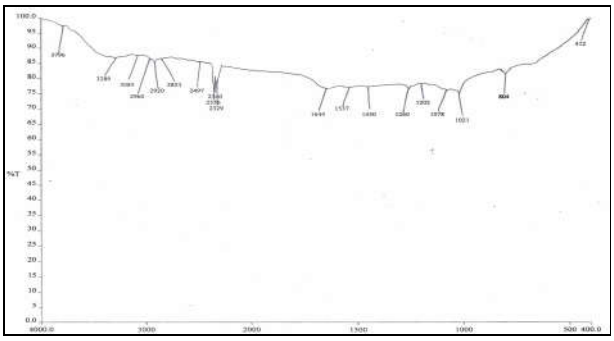


Fig. 3. Showing the FT-IR spectrum of standard collagen

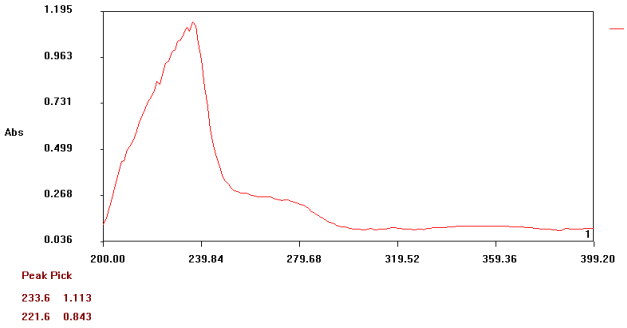


Fig. 5. UV – *vis* spectra of Pepsin – soluble collagen from *S. pharaonis*

UV – vis spectra

The UV-*vis* spectra of ASC and PSC of *S. pharaonis* are depicted in Fig. 4 & 5. The distinct absorbance of the ASC and PSC was obtained at 233.6nm, 236nm respectively.

are the main source of collagen for use in pharmaceutical, cosmetic and biomedical research. The aim for developing an alternative source of collagen is the infectious agent in the skin of bovine and porcine which can be transmitted to humans.

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

REGIONS	STANDARD	ASC	PSC	ASSINGMENT
Amide A	3289	3443	3420	NH stretch coupled with hydrogen bond
Amide B	2920	2923	2922	CH_2 asymmetrical stretch
-	2853	2853	2852	CH_2 asymmetrical stretch
Amide I	1644	1647	1649	C=O stretch/ hydrogen bond coupled with CN stretch
Amide II	1537	1541	1542	NH bend coupled with CN stretch
-	1450	1459	1459	CH_2 bend
-	-	1421	-	COO – symmetrical stretch
-	-	1336	-	CH_2 wagging proline
Amide III	1260	1241	1238	NH bend coupled with CN stretch
-	1078	1112	-	C-O stretch
-	1021	1023	1025	C-O stretch
-	804	-	-	Skeletal stretch
-	-	669	669	Skeletal stretch

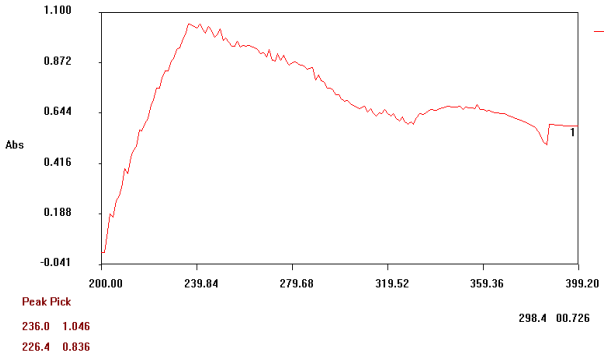


Fig. 4. UV – *vis* spectra of Acid – soluble collagen from *S. pharaonis*

SDS-PAGE

In SDS-PAGE, PSC of *S. pharaonis* showed distinct bands and lying between 73 kDa, 84 kDa and117 kDa. The ASC recorded only a single band and has molecular weight 107 kDa (Fig. 6).

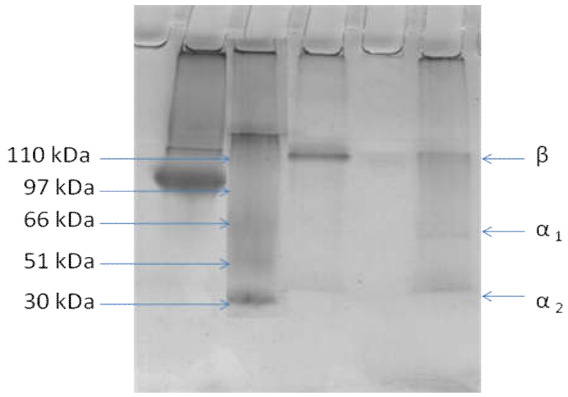
DISCUSSION

Collagen is the main constituent of the connective tissue of animal body. Collagen is a large super family having a large number of types of collagen exhibiting different structural and functional properties. The skin of vertebrates and invertebrates

This paved the way for the strong need to develop alternative source of collagen. The total protein contents of ASC and PSC from *S. pharaonis* were found to be 16.4% and 31.65% respectively. The result of present study was high when compare to the result of Mizuta et al. (2003) total protein content present in the arm and mantle of *O. vulgaris* as 9.1% and 14% respectively. The difference of the amount of total protein content of *S. pharaonis* may be due to whole body skin contains more protein when compare to arm and mantle of *O. vulgaris*.

Nagai *et al.* (2001) isolated 2% of ASC and 35% of PSC (on dry weight basis) from the skin of *Sepia lycidas* which is higher than that of the present findings. The ASC of the present study was found to be high when compared to the ASC (1.30%) of *Thysanoteuthis rhombus,* whereas the PSC of *S. pharaonis* is low when compare to the PSC (35.6%) of *T. rhombus* on dry weight basis reported by (Nagai, 2004). Nagai and Suzuki (2002) observed the yield of ASC (5.2%) and PSC (50%) from skin of paper nautilus *Argonauta argo* on dry weight basis. This is high as compared to collagen from *S. pharaonis*. 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. There are only few researches dealing with the practical utilization of connective tissue protein of sea vertebrates and invertebrates (Gudmundsson and Hafsteinsson, 1997; Guillen and Mentero, 2001). Type-I collagen is predominant in higher order animals

especially in the skin, tendon and bone where extreme forces are transmitted. It is a compound of three chains, two of which are identical, named as α1 and α2 chain with different amino acid composition or it can rarely expressed as trimer built of three α1 chains. Type II collagen is essentially unique to hyaline cartilage and the α2 subunit is believed to be similar to α1. Type III collagen is found in limited quantities (~10%) in association with type I collagen present in skin (Piez, 1985).



Lane 1 – Standard; Lane 2 – Marker; Lane 3 – ASC and Lane 4 – PSC

Fig. 6. SDS-PAGE profile of collagen from *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 (13220-1220 cm^{-1}) is related to CN stretching and NH and it is involved with the triple helix structure of collagen (Jakobsen *et al.,* 1983; Surewicz and Mantsh, 1988; Muyonga *et al.,* 2004). In the FT-IR studies of collagen from yellow fin tuna *Thunnus albacores* dorsal fin, Woo *et al.* (2008) observed the, amide region bands of A, I, II, and III at wavelengths of 3427 cm^{-1}, 1651 cm^{-1}, 1547 cm^{-1} and 1544 cm^{-1} respectively. In the present study, the amide region bands of Amide A, B, I, II and III are at 3443 cm^{-1}, 2923 cm^{-1}, 1647 cm^{-1}, 1541 cm^{-1} and 1241 cm^{-1} for ASC and 3420cm^{-1}, 2922 cm^{-1}, 1649 cm^{-1}, 1542 cm^{-1} and 1238 cm^{-1} for PSC respectively.

Plepis *et al.* (1996) observed that the triple helical structure of skin, scale and bone collagen of *S. mentella*, was confirmed by the peak present between 1240 cm^{-1} (Amide III) and 1454 cm^{-1} bands. FT-IR spectra of ASC from walleye pollock skin *Theragra calcogramma* exhibited peaks for amide A, amide B, I and III at 3328 cm^{-1}, 3080 cm^{-1}, 1648 cm^{-1} and 1236cm^{-1} respectively. The peaks of amide I and II of PSC (1649 cm^{-1} and 1542 cm^{-1} respectively) were at the same frequency as that of ASC (1647 cm^{-1} and 1541 cm^{-1} respectively) which indicates similarity in the molecular order of ASC and PSC. 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 (Payne and Veis, 1988) and was a sensitive marker of peptide secondary structure (Surewicz and Mantsch, 1988). The amide I band position was observed at 1647 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 I at 1241 cm^{-1} and 1459 cm^{-1} (ASC and PSC) confirms triple helical structure of collagen from the skin of *S. pharaonis*. The UV- *vis* spectra of ASC and PSC of *S. pharaonis* absorbance were obtained in (Fig. 4 & 5) 233.6 nm, 236 nm respectively. Generally, tyrosine and phenylalanine are sensitive chromophores and absorb UV light at 283 nm and 251 nm (Liu and Liu, 2006), where ASC and PSC has no evident absorbance. Therefore, ASC and PSC from *S. pharaonis* skin well support the property to collagen that there is absorbance at 230-240 nm, with little or no absorbance near 280 nm. Thus designate the protein is collagen.

The SDS-PAGE study revealed that electrophoretic pattern of ASC comprising α_1 was similar to PSC comprising α_1 and α_2 from *S. lycidas* as well as to porcine collagen (Nagai *et al.,* 2001); Whereas ASC obtained from skin of diamond squid *T. rhombus* consists of only one chain, α1 (Nagai, 2003). In the present study SDS-PAGE recorded the presence of one and two α chains in ASC and PSC respectively. The molecular weight of *S. pharaonis* skin was found to be 107 kDa in ASC and 117 kDa, 84 kDa and 73 kDa in PSC respectively (Fig. 6) Muyonga *et al.* (2004) and *Yan* et al. (2008) reported the ASC from Nile Perch skin and Walley Pollock skin by SDS-PAGE and reported that collagen consist of $\alpha_1$and α_2 which showed two distinct species varying in their mobility, their dimer (β chain). They concluded that existence of at least two different subunits showed that major collagen from Walley Pollock skin might be the type I collagen. This is accordant with the present study, observed the molecular weight between 73-117 kDa which is similar to that of type I collagen.

Conclusion

The collagen (ASC and PSC) was extracted from the outer skin of cuttlefish *S. pharaonis* and characterized. The FT-IR investigation showed the existence of helical arrangement of collagen. Therefore from the results of the present study, it may be inferred that there is a possibility to use skin waste of cuttlefish sea food from the processing plants as an alternative source for the present may conventional source of collagen for industrial purposes.

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