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Isolation and Characterization of Collagen from Squid (*Ommastrephes bartrami*) Skin

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Abstract Collagen of squid (*Ommastrephes bartrami*) skin was examined in the present study. Histology showed that collagen fiber in the skin was partially cross-linked with muscle fiber. Acid-solubilized collagen (ASC) and pepsin-solubilized collagen (PSC) were extracted from the skin and characterized. The results of amino acid composition and electrophoretic patterns revealed that ASC and PSC were both type I collagen, containing α1 and α2 chains. FTIR (fourier transform infrared spectroscopy) investigations confirmed the existence of helical arrangements in PSC of squid skin. The denaturation temperature (T_d) and shrinkage temperature (T_s) of PSC were 29.4℃ and 52.8℃, respectively.

Key words isolation; characterization; collagen; thermal stability; squid

1 Introduction

Collagen is an abundant protein in animal tissues and constitutes approximately 30% of the total body protein (Muyonga *et al*., 2004). It has a wide range of applications in leather and film industries, pharmaceutical, cosmetic and biomedical materials, and food industry (Kittiphattanabawon *et al*., 2005). Examples are production of wound dressings, vitreous implants and carriers for drug delivery, edible casings (Senaratne *et al*., 2006) and production of cosmetics with good moisturizing properties (Swatschek *et al*., 2002). So far, the main sources of collagen for industrial use have been limited to land-based animals, such as bovine or porcine skin and bone. However, the outbreak of bovine spongiform encephalopathy (BSE) and foot-and-mouth disease (FMD) crisis in recent decades have raised concerns among consumers over collagen and collagen-derived products of land-animal origin (Jongjareonrak *et al.*, 2005). In addition, the collagen extracted from porcine is not suitable for use as a component in some foods due to social and cultural concerns. Therefore, alternative sources of collagen should be developed. Researchers have found that the skin, bone, scale, fin and cartilage of freshwater and marine fish, the mantle of scallops (Shen *et al.*, 2007), the muscle layer of ascidians (Mizuta *et al.*, 2002a), and the adductor of pearl oysters (Mizuta *et al.*, 2002b) can be used as new sources of collagen.

Squid (*Ommastrephes bartrami*) is one of the commercially important fish species in China. At present, approximately 300−400 thousand tons of squid are processed per year, mainly in Shandong and Zhejiang Provinces. Approximately 8−10 percent of the leftovers are squid skin, being considered as wastes in the fish shops and refrigerated fish processing factories (Lin and Li, 2006). If the skin is to be dumped as wastes as is the case currently, this would pose a potential threat to the environment (*e.g.* pollution and offensive odor). Research has shown that about 70% of the squid skin dry matter is collagen (Zhang *et al.*, 2003). To make the skin as an effective source of collagen, it is necessary to obtain fundamental information about the squid skin collagen. The present paper describes the isolation and physical-chemical properties of the collagen from the squid (*Ommastrephes bartrami*) skin.

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2 Materials and Methods

2.1 Fish Skin Preparation

Squid (*Ommastrephes bartrami*) skins were collected from a local fish processing factory in Qingdao. They were brought to our laboratory and stored at –20℃ until used. The skins were descaled and the residual meat was removed manually. All the reagents used in the experiment were of analytical grade.

2.2 Histological Observation

Histological observation was carried out essentially as described by Zhao *et al.* (2007). Squid skin was cut into pieces (0.5 cm × 0.5 cm) and fixed in 4% buffered formalin over 24 h. The specimen was then dehydrated in a series of graded ethanol solutions (70%, 80%, 90%, and 100%), clarified in xylene and finally embedded in paraffin.

Eight-micron sections were cut perpendicular to the skin surface. The slides (with the sections) were cleared off paraffin with xylene, rehydrated to water through graded alcohols (100%, 90%, 80%, and 70%), and stained with hematoxylin and eosin and further with the Van Gieson stain (collagen, red color; muscle, yellow color). The stained sections were observed using light microscopy. Digital images were obtained using a color video camera (Olympus BX51, Olympus Optical Co. Ltd., Tokyo, Japan) at an original magnification of 20×.

2.3 Extraction of Collagen

The extraction procedures were performed at 4℃ as described by Nagai and Suzuki (2000), but with slight modifications. The skin was extracted with 0.1 molL^{-1} NaOH to remove non-collagenous materials and exclude the effect of endogenous proteases on collagen (Sato *et al.*, 1987), and rinsed with distilled water thoroughly until the pH of the wash water became neutral. The samples were then defatted with 10% butyl alcohol at a solid to solvent ratio of 1:10 for 24 h, and washed with ample amount of distilled water. The further minced skins were gently stirred in 0.5 molL^{-1} acetic acid solution for 48 h, and the extract was centrifuged at 10000×g for 30 min. Then the acid-solubilized collagen (ASC) in the supernatant was salted out by adding NaCl to a final concentration of 0.9 molL^{-1}. After the solution being left overnight, the resultant precipitate was collected by centrifugation at 8000×g for 20 min and then dissolved in 0.5 molL^{-1} acetic acid, followed by being dialyzed against 0.1 molL^{-1} acetic acid for 1 d, and distilled water for 2 d, and lyophilized.

After acid extraction, the insoluble fraction was suspended in 10 times of 0.5 molL^{-1} acetic acid (by volume) and digested with porcine pepsin (EC 3. 4. 23. 1; powderized; 750 U mg^{-1} dry matter, Sigma, USA) at an enzyme/substrate ratio of 1:100 for 48 h at 4℃ with gently stirring. The extract was centrifuged at 10000×g for 30 min. Then the pepsin-solubilized collagen (PSC) in the supernatant was salted out by adding NaCl to a final concentration of 0.9 molL^{-1}. The solution was left overnight, and the resultant precipitate collected by centrifugation (8000×g for 20 min) was dissolved in 0.5 molL^{-1} acetic acid, dialyzed against 0.02 molL^{-1} Na_2HPO_4 for 1 d to inactivate pepsin. The precipitate was collected at low speed centrifugation and dissolved in 0.5 molL^{-1} acetic acid. Then the solution was dialyzed with 0.1 molL^{-1} acetic acid and distilled water in the same way as for ASC preparation, and lyophilized.

2.4 Amino acid Analysis

ASC and PSC samples were hydrolyzed under reduced pressure with 6 molL^{-1} HCl at 110℃ for 24 h. The hydrolysates were analyzed on a Hitachi 835-50 amino acid analyzer (Hitachi, Tokyo, Japan).

2.5 Sodium Dodecyl Sulphate Polyacylamide Gel electrophoresis (SDS-PAGE)

SDS-PAGE was performed by following the method of Laemmli (1970), using the discontinuous Tris-HCl/glycine buffer system with 7.5% resolving gel and 5% stacking gel. After electrophoresis, the gel was stained for 20 min with 0.1% Coomassie Brilliant Blue R-250 dissolved in distilled water, methanol and acetic acid (9:9:2), and then destained using a solution containing distilled water, methanol and acetic acid (8:1:1).

2.6 Fourier Transform Infrared Spectroscopy (FTIR)

FTIR spectra were obtained from samples placed on discs containing a mixture of 0.2 mg lyophilized collagen and about 10 mg potassium bromide (KBr) ground under drying conditions. The spectra were recorded using infrared spectrophotometer (Nicolet 200SXV, Wisconsin, USA) from 4000 to 500 cm^{-1} at a data acquisition rate of 2 cm^{-1} per point. The resulting spectra were analyzed using the Omnic 6.0 software (Thermo-Nicolet, Madison, Wisconsin, USA).

2.7 Determination of Denaturation Temperature

The denaturation temperature was measured from changes in viscosity, using an Ubbelohde viscometer, according to the method modified from Zhang *et al.* (2007). Briefly, ten milliliters of 0.03% collagen solution in 0.1 molL^{-1} acetic acid with 0.2 molL^{-1} sodium acetate buffer (pH 5.0) were used for viscosity measurements. Thermal determination curve was obtained by measuring the solution viscosity at seven stepwise-raised temperatures from 16 to 42℃, each temperature being maintained for 30 min. Fractional viscosity at a given temperature was calculated by the following equation:

$$\text{Fractional viscosity}=(\eta_{sp(T)}-\eta_{sp(42℃)})/(\eta_{sp(16℃)}-\eta_{sp(42℃)}),$$

where η_{sp} is the specific viscosity. These fractional viscosities were plotted against the temperature and the denaturation temperature was determined as the temperature where the fractional viscosity was predicted to be 0.5.

2.8 Differential Scanning Calorimetry

Differential scanning calorimetry (DSC) was performed on a Netzsch DSC 200PC calorimeter (Netzsch, Bavaria, Germany) fitted with an air cooling compressor and a liquid nitrogen cooler at ambient temperature (Cui *et al.*, 2007). The temperature was calibrated using indium as standard. Collagen fiber was weighed (3.00 mg) and sealed in aluminium pans (BO 6.239.2−64.502). Triplicate samples were heated from 20 to 100℃ at a scanning rate of 2 K min^{-1}, with an empty sealed pan as a reference. The shrinkage temperature was taken at the peak of the plotted thermal transition curve.

3 Results and Discussion

3.1 Distribution and Isolatioin of Collagen in Squid Skin

As shown in Fig.1, collagen fiber was observed in the muscle layer surrounding the muscle fiber partially. It was

also observed in the epidermis which is an outer sheath of squid skin. This cross-linking with muscle fiber may explain that collagen in squid skin was not completely solubilized with 0.5 molL^{-1} acetic acid. The residues were re-extracted with pepsin, and the collagen of squid skin was easily solubilized by pepsin proteolysis. With further digestion by limited pepsin, the cross-linked molecules at the telopeptide region were cleaved but without damaging the integrity of the triple helix (Jongjareonrak *et al.*, 2005). Yields of ASC and PSC isolated from squid skin were 36.2% and 16.4% (dry weight basis), respectively. Therefore, the major fraction of squid skin collagen was ASC. The content of ASC fraction in squid skin was different from those reported in cuttlefish skin (2.0%) (Nagai *et al.*, 2001), ocellate puffer fish skin (10.7%) (Nagai *et al.*, 2002), and grass carp skin (8%) (Zhang *et al.*, 2007).

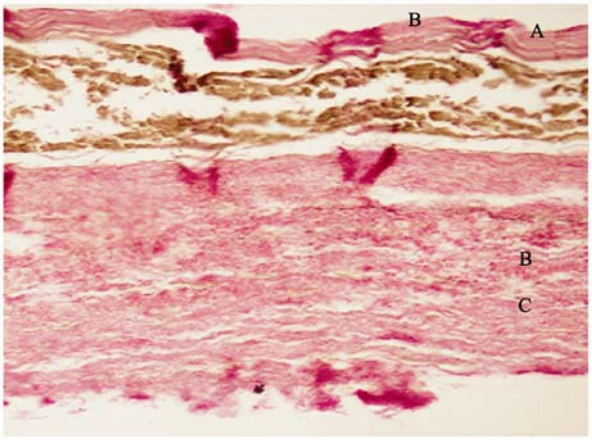

Fig.1 Light micrographs of the squid skin stained with Van Gieson stain (*magnification* ×20). Collagen fibers and muscle fibers were stained with red and yellow colors, respectively. A. Epidermis; B. Collagen fiber; C. Muscle fiber.

3.2 Amino Acid Composition

The amino acid composition of ASC and PSC from squid skin, expressed as residues per 1000 total residues, is shown in Table 1. It was observed that ASC and PSC extracted from squid skin had similar amino acid profiles with glycine being the major amino acid (about 23.9% and 24.3%, respectively). Similar results were obtained in brownstripe red snapper skin (Jongjareonrak *et al.*, 2005). However, both ASC and PSC were found to contain low levels of histidine (both 0.9%), tyrosine (about 1.4% and 1.6%, respectively), lysine (about 1.8% and 1.4%, respectively) and isoleucine (about 1.8% and 1.6%, respectively). The imino acid (proline and hydroxyproline) contents of ASC and PSC from squid skin were 18.0% and 18.7%, respectively, close to that of 18.6% in grass carp collagen (Zhang *et al.*, 2007), but lower than that of 22% in porcine skin collagen (Ikoma *et al.*, 2003) and 21.5% in bovine skin collagen (Cui *et al.*, 2007). Hydroxyproline was derived from proline by post-translational hydroxylation mediated by prolylhydroxylase (Li *et al.*, 2004). The degree of hydroxylation of proline residues in ASC and PSC from squid skin were 46.7% and 47.6%, respectively, which were close to that of 48% reported for cuttlefish skin (Nagai *et al.*, 2001), but higher than that of 39% for ocellate puffer fish skin (Nagai *et al.*, 2002) and 43% for channel catfish skin (Liu *et al.*, 2007). The amino acid composition indicated that ASC and PSC from squid skin might be type I collagen.

Table 1 Amino acid composition of collagen from squid skin (amino acid residues/1000 total amino acid residues)

Amino acid	Acid-solubilized collagen (ASC)	Pepsin-solubilized collagen (PSC)
Hydroxyproline	84	89
Aspartic acid	74	73
Threonine	27	27
Serine	36	37
Glutamic acid	116	120
Glycine	239	243
Alanine	62	63
Valine	34	32
Methionine	29	25
Isoleucine	18	16
Leucine	35	30
Tyrosine	14	16
Phenylalanine	22	17
Lysine	18	14
Histidine	9	9
Arginine	87	91
Proline	96	98

3.3 Sodium Dodecyl Sulphate Polyacylamide Gel Electrophoresis (SDS-PAGE)

The collagen from squid skin was examined by SDS-PAGE using a 7.5% resolving gel (Fig.2). Both ASC and PSC had the similar electrophoretic pattern of typical type I collagen consisting of α chains with two distinct types (α1 and α2 chains) varying in their mobility. The electrophoretic positions of α chains of squid skin collagen (115 kDa for α1 chain, and 66 kDa for α2 chain) were different from those of the skin collagens of walleye pol-

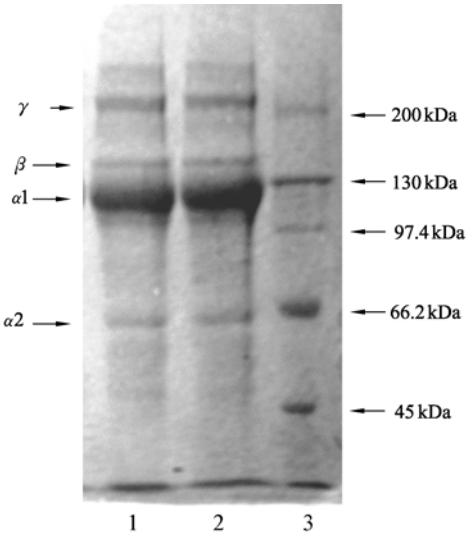

Fig.2 SDS-PAGE patterns of collagen from squid skin. Lane 1: pepsin-solubilized collagen (PSC); lane 2: acid-solubilized collagen (ASC); lane 3: protein markers.

lock (Yan *et al.*, 2008), grass carp, bovine (Zhang *et al.*, 2007) and porcine, indicating that α chains of squid skin collagen were distinct in its primary structure. Moreover, the inter and intra molecular crosslinked components, β (dimers) and γ (trimers), were also found in squid skin collagen, which was similar to those of bigeye snapper (Kittiphattanabawon *et al.*, 2005) and ocellate puffer fish skin (Nagai *et al.*, 2002). The electrophoretic results further showed that the major collagen from squid skin was type I collagen.

3.4 Fourier Transform Infrared Spectroscopy

Fig.3 shows the FTIR spectra of the pepsin-solubilized collagen from squid skin, with patterns similar to those exhibited by other collagens (Muyonga *et al.*, 2004; Liu *et al.*, 2007). The amide A band was associated with the N-H stretching frequency. A free N-H stretching vibration occurred in the range of 3400−3440 cm^{-1}, and when the NH group of a peptide was involved in a hydrogen bond, the position was shifted to lower frequencies (Li *et al.*, 2004). The amide A band of squid skin collagen was found at 3224 cm^{-1}, suggesting an involvement of the hydrogen bond. However, this value was lower than those of channel catfish skin collagen (3330 cm^{-1}) (Liu *et al.*, 2007), and walleye pollock skin collagen (3328 cm^{-1}) (Yan *et al.*, 2008). The amide B band of squid skin collagen was found at 3052 cm^{-1}, and it is related to the asymmetrical stretch of CH_2 (Muyonga *et al.*, 2004).

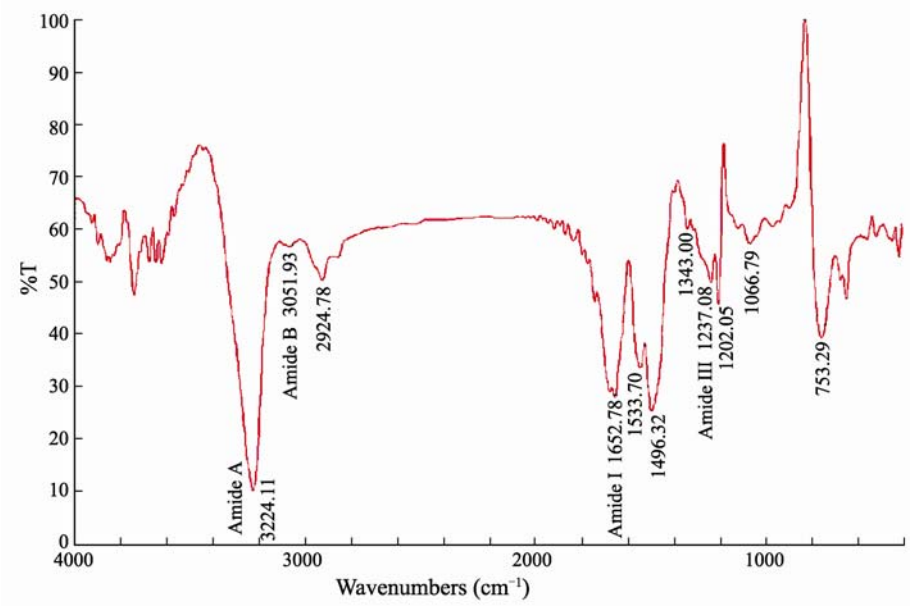

Fig.3 Fourier transform infrared spectra (FTIR) of pepsin-solubilized collagen (PSC) from squid skin.

The position of amide I band (the absorption band of C=O stretching) was observed at 1652 cm^{-1}. This band is associated with the secondary structure of the protein. The absorption between the 1237 cm^{-1} (amide Ⅲ) and 1496 cm^{-1} band observed in squid skin collagen affords evidence of the existence of helical structure (Liu *et al.*, 2007).

3.5 Thermal Stability

Thermal stability of collagen is usually described by the denaturation temperature (T_d) in solution and the shrinkage temperature (T_s) of fiber. The temperature at which the triple helix structure of collagen is disintegrated into random coils is considered as T_d (Hao and Li, 1999). Fig.4 shows the thermal denaturation curve of squid skin collagen. T_d of squid skin collagen was 29.4℃, close to those of collagens from skins of ocellate puffer fish (28.0℃) (Nagai *et al.*, 2002), grass carp (28.4℃) (Zhang *et al.*, 2007), and brown backed toadfish (28.0℃) (Senaratne *et al.*, 2006), higher than that of cod skin collagen (15.0℃) (Rigby, 1968), but about 8℃ lower than that of collagen from porcine skin (37.0℃) (Nagai *et al.*, 1999). The shrinkage temperature refers to the temperature at which collagen fiber shrinks to one third of its length (Fathima *et al.*, 2004). During the shrinkage process, a phase transition involves the conversion of a crystalline triple helical collagen structure to an amorphous random coil form (Fathima *et al.*, 2003). T_s of collagen from squid skin was found to be at 52.8℃ (Fig.5), about 9℃ lower than that of type I collagen from bovine skin (62.0℃) and lower than that of PSC from the body wall of sea cucumber (57.0℃) (Cui *et al.*, 2007). These results showed the helices of collagen from squid skin were less stable than those of mammalian collagens. We also found DSC thermogram was rather broad, reflecting smaller cooperatives among the participation subunits (Usha and Ramasami, 2004). The difference between T_s and T_d of squid skin collagen was about 23.4℃, which was consistent with the conclusion that the difference between T_s and T_d of marine collagen was not much influenced by species (20−25℃) (Hao and Li, 1999).

The thermal stability is influenced by the imino acid content, the higher the imino acid content, the more stable the helices, for the molecular structure of collagen is maintained mainly by restrictions on changes in the sec-

ondary structure of the polypeptide chain (imposed by the pyrrolidine rings of proline and hydroxyproline), and also maintained partially by the hydrogen bond ability through the hydroxy group of hydroxyproline (Zhang *et al.*, 2007). Therefore, the helices of ASC and PSC from squid skin with lower imino acid contents (180 and 187 residues per 1000 residues, respectively) denatured at lower temperatures, compared with porcine and bovine skin collagen having higher imino acid contents (220 and 215 residues per 1000 residues, respectively) (Ikoma *et al.*, 2003; Cui *et al.*, 2007). On the other hand, the stability of collagen is also known to be correlated with the environmental and body temperatures (Rigby, 1968). The imino acid contents were close between squid skin collagen and walleye pollock skin collagen (Yan *et al.*, 2008), so the higher denaturation temperature of squid skin collagen may be attributed to the environmental and body temperatures.

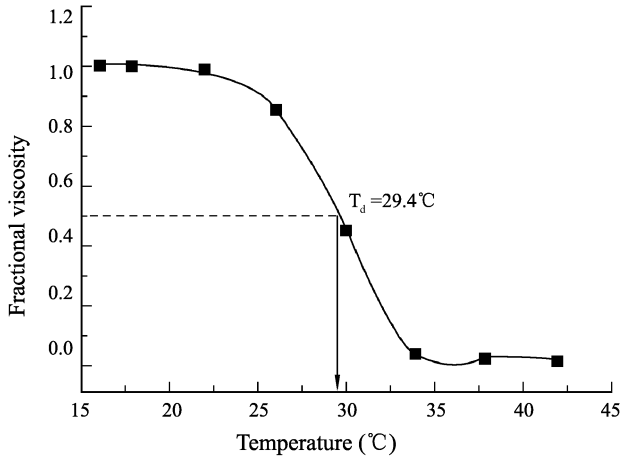

Fig.4 Thermal denaturation curve of pepsin-solubilized collagen (PSC) from squid skin.

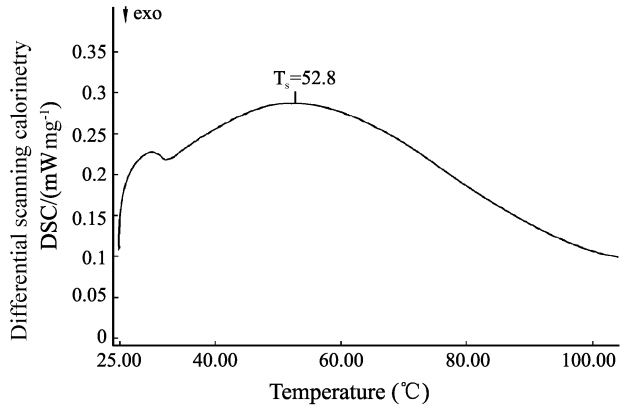

Fig.5 Thermal transition curve of pepsin-solubilized collagen (PSC) from squid skin, as shown by DSC.

4 Conclusion

ASC and PSC were extracted from squid skin. The results of amino acid composition and electrophoretic patterns revealed that the ASC and PSC were both type I collagen, containing α1 and α2 chains. FTIR investigations showed evidence of helical arrangements of PSC. The denaturation temperature (T_d) and shrinkage temperature (T_s) of PSC were 29.4℃ and 52.8℃, respectively.

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