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Preparation and partial characterization of collagen from paper nautilus (*Argonauta argo,* Linnaeus) outer skin

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Abstract

To make more effective use of limited-resources, collagen was prepared from the outer skin of the paper nautilus. As a result, the outer skin was hardly solubilized in 0.5 M acetic acid (yield: about 5.2% on a dry weight basis). The insoluble matter was easily digested by 10% pepsin (w/v), and a large amount of collagen was obtained with about a 50.0% yield (pepsin-solubilized collagen). The pepsin-solubilized collagen had a chain composition of α1 α2 α3 heterotrimer similar to *Callistoctopus arakawai* arm collagen. Although the paper nautilus collagen has a denaturation temperature of 27 °C, that is about 10 °C lower than that of the porcine one, this result indicates that it is possible to use the paper nautilus outer skin as an important collagen source.

Keywords: Paper nautilus; Outer skin; Collagen; Yield

1. Introduction

The paper nautilus, *Argonauta argo,* belongs to the order Octopoda, and is a member of the octopus family. The sizes of the male and female are quite different (male: about 1.5 cm; the female: about 30 cm). It is unique among the octopus species and, specifically, the female paper nautilus has a mallow-type shell. It is distributed in the East China Sea and surrounding waters.

The Japanese consume octopus as sliced raw flesh, a vinegared dish, smoked fish, Japanese hotchpotch, and flavouring processed foodstuffs and cooked food. However, the species consumed in Japan are limited. Octopus species such as *Eledone moschata, E. cirrosa, Octopus briareus, O. maya, O. tetricus,* and *Cistopus indicus* are consumed worldwide (Tanaka, Okutani, Arakawa, & Hamada, 1999).

There have been many reports of collagen in marine organisms (Kimura, Miura, & Park, 1983; Kimura & Ohno, 1987; Kimura, Omura, Ishida, & Shirai, 1993; Kimura, Takema, & Kubota, 1981; Kimura, Zhu, Matsui, Shijoh, & Takamizawa, 1988; Miura & Kimura, 1985; Nomura, Sakai, Ishii, & Shirai, 1996, Shimizu, Amemiya, & Yoshizato, 1990, Trotter & Koob, 1994). Nagai and co-workers have also investigated it from the viewpoint of an underutilized fisheries resources (Nagai, Nagamori, Yamashita, & Suzuki, 2001; Nagai, Ogawa, Nakamura, Ito, Nakagawa, Fujiki, Nakao, & Yano, 1999; Nagai & Suzuki, 1999, 2000a,b; Nagai & Suzuki, 2001; Nagai, Worawattanamateekul, Suzuki, Nakamura, Ito, Fujiki, Nakao, & Yano, 2000). From previous studies, it was found that the collagen could be easily obtained, in a good yield, from fisheries resources (Nagai et al., 1999, 2000, 2001; Nagai, Nagamori, et al., 2001; Nagai & Suzuki, 1999, 2000a,b).

Collagen is a protein of high content in the living body. The main sources of industrial collagen are limited to pig and bovine skin and bones. If a great amount of collagen can be obtained from paper nautilus, it has potential use as an alternative to mammalian collagen in foods, cosmetics and biomedical materials. Recently, there has been interest in investigating possible means for making more effective use of underutilized resources and industrial wastes. This paper deals with the preparation and the partial characterization of collagen from the paper nautilus outer skin and compares its characteristics with the collagen from other octopi.

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2. Materials and methods

2.1. Sample

The paper nautilus (*Argonauta argo,* Linnaeus) was caught in Hibiki Nada, Shimonoseki, Yamaguchi, Japan. Their outer skins were excised, washed with distilled water, cut into small pieces, and stored at −85 °C until used.

2.2. Preparation of collagen from outer skin

All the preparative procedures were performed at 4 °C. To remove the noncollagenous proteins and pigments, the skins were extracted with 0.1 M NaOH for 2 days, then washed with distilled water, and lyophilized. The lyophilized matter was extracted with 0.5 M acetic acid for one day, and the extract was centrifuged at 50,000×***g*** for 1 h. The residue was re-extracted with the same solution for one day, and the extracts were centrifuged under the same conditions. Each solution was mixed and salted out by adding NaCl to a final concentration of 0.7 M, followed by precipitation of the collagen by the addition of NaCl (final concentration of 2.3 M) in 0.05 M Tris-HC1, pH 7.5. The resultant precipitate was obtained by centrifugation at 50,000×***g*** for 1 h, dissolved in 0.5 M acetic acid, dialyzed against 0.1 M acetic acid, distilled water and then lyophilized (acid-solubilzed collagen; ASC).

The insoluble fraction was suspended in 0.5 M acetic acid, then digested with 10% (w/v) pepsin (EC 3. 4. 23. 1; 2× crystallized; 3,085 U/mg protein, Sigma, USA) at 4 °C for 2 days. The pepsin-solubilized collagen (PSC) was centrifuged at 50,000×**g** for 1 h and the supernatants were dialyzed to inactivate the pepsin against 0.02 M Na_2HPO_4 (pH 7.2) for 3 days with a change of solution once a day. The precipitate obtained by centrifugation at 50,000×*g* for 1 h was dissolved in 0.5 M acetic acid and salted out by adding NaCl to a final concentration of 0.7 M, followed by precipitation of the collagen by the addition of final concentration of 2.3 M NaC1 in 0.05 M Tris-HC1, pH 7.5. The resultant precipitate was obtained by centrifugation at 50,000×***g*** for 1 h, dissolved in 0.5 M acetic acid, dialyzed against 0.1 M acetic acid, and then lyophilized.

2.3. SDS-polyacrylamide gel electrophoresis (SDS-PAGE)

SDS-PAGE was performed as previously described (Nagai, Yamashita et al., 2001). The collagen sample was dissolved in 0.02 M sodium phosphate buffer (pH 7.2), containing 1% SDS and 3.5 M urea at 4 °C for 30 min. Electrophoresis was performed on 3.5% gels in 0.1 M sodium phosphate buffer (pH 7.2) containing 0.1% SDS. After the electrophoresis, each gel was stained with Coomassie Brilliant Blue R-250 (Fluka Fine Chemical Co., Ltd., Tokyo, Japan) and destained with 5% methanol and 7.5% acetic acid.

2.4. Peptide mapping

A collagen sample (1.0 mg) was dissolved in 0.1 M sodium phosphate buffer (pH 7.2) containing 0.5% SDS and heated at 100 °C for 5 min. The digestion was carried out at 37 °C for 30 min by adding 5 μl of lysyl endopeptidase from *Achromobacter lyticus* (EC 3. 4. 21. 50; 4.5 amidase activity/mg protein; Wako Pure Chemicals, Osaka, Japan). After adding SDS to a final concentration of 2%, the proteolysis was stopped by boiling for 5 min. SDS-PAGE was performed by the method of Laemmli (1970) using a 15% gel.

2.5. CM-Toyopearl 650M column chromatography

The subunit components of this collagen were separated by using CM-Toyopearl 650M (Tosoh Co., Tokyo, Japan) column chromatography. The collagen sample (15.0 mg) was dissolved in 5 ml of 20 mM sodium acetate buffer (pH 4.8) containing 6 M urea at 4 °C and denatured at 45 °C for 30 min. After centrifugation at 50,000×***g*** and room temperature for 30 min, the denatured collagen sample was applied to a CM-Toyopearl 650M column (1.0×5.0 cm) previously equilibrated with the same buffer. Elution was achieved with a linear gradient of 0–0.15 M NaC1 in the same buffer at a flow rate of 0.9 ml/mm. Each subunit was detected by monitoring the absorbance at 230 nm.

2.6. Denaturation temperature (Td)

Td as measured by the method of Nagai, Yamashita et al. (2001). Five millilitres of the 0.03% collagen solution in 0.1 M acetic acid were used for the viscosity measurements, and measurement was done using a Canon-Fenske type viscometer with an average shear gradient of 400 s^{-1}. The thermal determination curve was obtained by measuring solution viscosity at several temperatures from 10 to 50 °C; the temperature was raised stepwise and maintained for 30 mm. The Td was determined as the temperature at which the change in viscosity was 50% completed. Each point is the mean of triplicate determinations.

3. Results and discussion

3.1. Isolation of collagen from paper nautilus outer skin

The skins were hardly solubilized using 0.5 M acetic acid. The ASC yield was about 5.2% on a dry weight basis and was very low. By digestion with pepsin, PSC

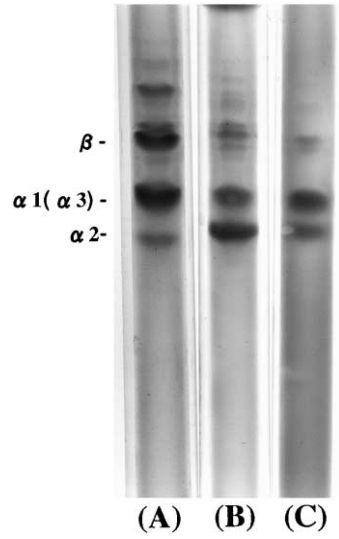

Fig. 1. SDS-polyacrylamide gel electrophoresis of porcine skin type I collagen and paper nautilus outer skin collagen on 3.5% gels containing 3.5 M urea. (A) Porcine skin collagen; (B) paper nautilus outer skin acid-solublized collagen; (C) paper nautilus outer skin pepsin-solubilized collagen.

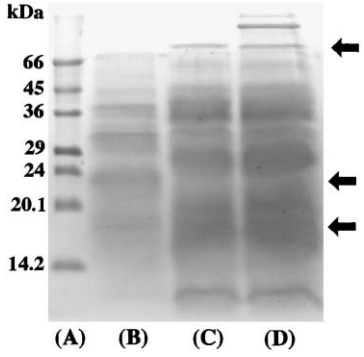

Fig. 2. Peptide mapping of lysyl endopeptidase digests from paper nautilus outer skin collagens. (A) Molecular weight marker; (B) porcine skin collagen; (C) paper nautilus outer skin acid-solublized collagen; (D) paper nautilus outer skin pepsin-solubilized collagen.

was perfectly solubilized. PSC was effectively precipitated and purified with salts of different concentrations. The PSC yield was very high and was about 50.0% on a dry weight basis. By using 3.5% SDS-PAGE, ASC and PSC showed two α chains, corresponding to α1 and α2 (Fig. 1). Moreover, a small amount of the β chain was obtained in these collagens. Kimura et al. (l981) reported the isolation and characterization of pepsin-digested collagen from the octopus *O. vulgaris* skin. The *O. vulgaris* skin collagen was readily precipitated at approximately 0.4 M NaCl, that is about one-half the NaCl concentration for the paper nautilus outer skin collagen.

3.2. Peptide mapping

The denatured collagens were examined by SDS-PAGE in order to directly compare the pattern of peptide

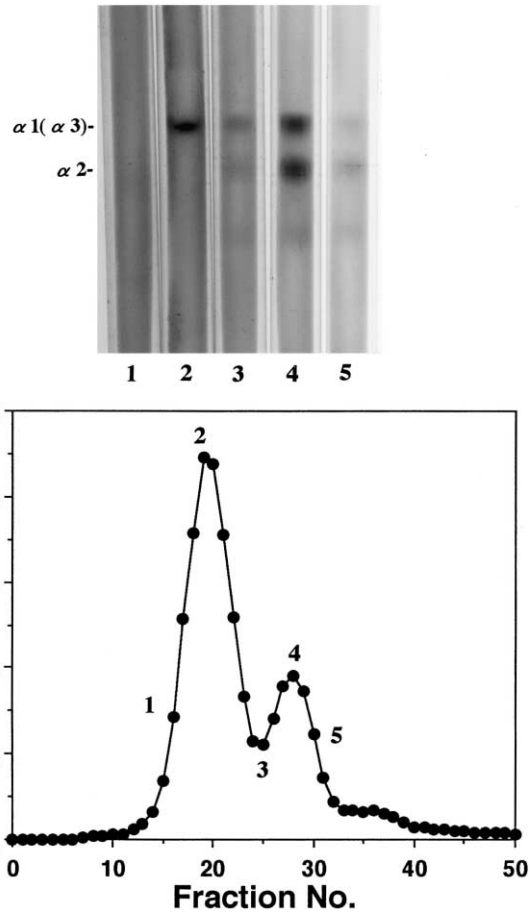

Fig. 3. CM-Toyopearl 650M column chromatography of denatured paper nautilus outer skin collagen. A 1.0×5.0 cm column of CM-Toyopearl 650M was equilibrated with 20 mM sodium acetate buffer (pH 4.8) containing 6M urea, and maintained at 37 °C. The collagen sample (15.0 mg) was dissolved in 5 ml of the same buffer, denatured at 45 °C for 30 mm, and then eluted from the column with a linear gradient of 0 to 0.15 M NaCl at a flow rate of 0.9 ml/min. The fractions indicated by the numbers were examined by SDS-PAGE.

fragmentation with the porcine skin collagen. These collagens gave very similar, but not identical peptide maps (Fig. 2). Moreover, these patterns were different from that of porcine collagen. This result revealed that the peptide fragments of these collagens were fairly different from that of the porcine skin.

3.3. Subunit composition

The denatured PSC was fractionated by CM-Toyopearl 650M column chromatography. As a result, it was resolved into two peaks, one having a large absorbance and the other small (Fig. 3). To identify each α chain, several chromatographic fractions, as indicated by the numbers, were analyzed by SDS-PAGE. These results suggest that PSC consists of three α chains. An α1 chain was found in Fractions 18 to 22, as indicated by the number. An α2 chain was found in Fractions 25 to 31. Moreover, an α3 chain was also found in Fractions

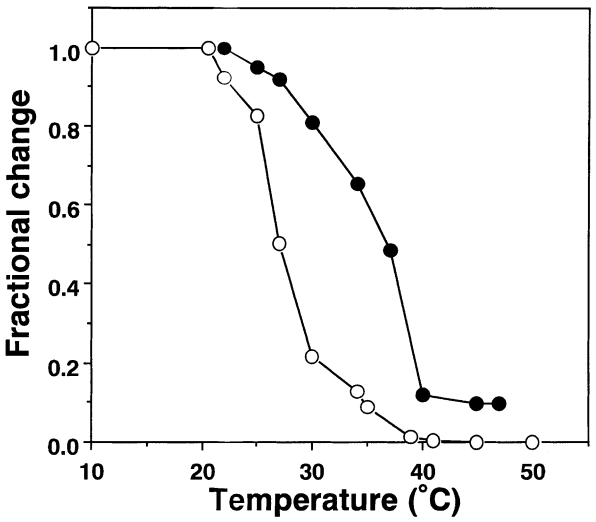

Fig. 4. Thermal denaturation curve of paper nautilus outer skin collagen solution as measured by viscosity in 0.1 M acetic acid. The incubation time at each temperature was 30 mm. Collagen concentration: 0.03%; ●: porcine skin collagen, ○: paper nautilus outer skin pepsin-solubilized collagen.

25 to 31. It is suggested that this collagen is a heterotrimer with a chain composition of α1 α2 α3. This result is similar to that from *Callistoctopus arakawai* arm (Nagai, Nagomori et al., 2001). Kimura et al. (1981) reported that the major collagenous component from the limited pepsin digests of the octopus skin had two distinct subunits, α1 and α2, which had the chain composition $(\alpha1)_2$ α2. On the other hand, Kimura et al. (1988) examined fish skin collagen and reported that the α3 chain was widely distributed in the teleost. In previous papers (Kimura, 1985; Kimura & Ohno, 1987; Kimura et al., 1987; Piez, 1965), it was reported that an α3 chain was detected in 14 fish species of 17 teleosts. The results of our present and previous papers (Nagai et al., 1999) were similar to those of many past papers. Therefore, it seems that the α3 chain is widely distributed, not only in the teleost skin, but also in marine invertebrate skins.

3.4. Denaturation temperature

The Td of this collagen sample was calculated from the thermal denaturation curves. As a comparison, the Td of porcine skin collagen was measured under the same conditions. The paper nautilus had a Td of 27.0 °C that was lower by about 10 °C than that of the porcine skin collagen (37.0 °C; Fig. 4). This value was the same as that obtained from other marine organisms (16.8–31.7 °C; Kimura & Ohmo, 1987; Kimura et al., 1988, 1993; Nagai, Nagamori et al., 2001; Nagai & Suzuki, 1999, 2000a; Nagai & Suzuki, 2001; Nagai et al., 1999, 2001). Rigby (1968) reported that the stability of collagen is correlated to environmental and body temperatures. This tendency is applicable to each of the results that were obtained from the previous papers (Kimura & Ohno, 1987; Kimura et al., 1988, 1993, Nagai, Nagamori et al., 2001, Nagai & Suzuki, 1999, 2000a; Nagai & Suzuki, 2001; Nagai et al., 1999, 2000).

In conclusion, the extractive content of the octopus species is rich in betaine, taurine and proline, similar to red-meat fish and squid. Therefore, the nutritional value of octopus is fairly high. The Japanese consume octopus such as *O. vulgaris, O. ocellatus, O. minor,* and *Paroctopus dofleini* by choice but do not eat other species. In this study, it was found that a great amount of collagen can be obtained from the paper nautilus outer skin (ASC, 5.2%; PSC, 50.0%). This result showed that it is possible to use the paper nautilus skin as an important collagen source for non-food applications.

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