Collagen from Diamondback Squid (*Thysanoteuthis rhombus*) Outer Skin

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Collagens (acid-solubilized and pepsin-solubilized collagens) were prepared from diamondback squid outer skin and partially characterized. The yields of acid-solubilized and pepsin-solubilized collagens were about 1.3 and 35.6%, respectively, on a dry weight basis. Pepsin-solubilized collagen was heterotrimer with a chain composition of $\alpha 1\alpha 2\alpha 3$. The patterns of peptide fragments were different from that of porcine skin collagen. Denaturation temperature was 27.5 °C, about 10 °C lower than that of porcine collagen. The amino acid composition of pepsin-solubilized collagen from diamondback squid outer skin was similar to that from cuttlefish outer skin. This squid is big among squid species, and its skin is thick. It is clear that diamondback squid outer skin has a potential as an alternative source of collagen to bovine skin and bone. At present, collagen using aquatic materials such as skin (cod and a deep-sea fish) and scale (sea bream and anchovy) is the development stage in the related industries. Unless the problem of BSE infection in land animals is resolved aquatic materials as an alternative source of collagen will attract much attention in the cosmetic and medical fields.

Key words: Collagen, Diamondback Squid, Alternative Source of Mammalian Collagen

Introduction

Collagen is a protein which is found in the highest concentration, about 30%, in the living body. The main sources of industrial collagen are limited to those from bovine and pig skin and bones. However, the existence of bovines infected with Bovine Spongiform Encephalopathy (BSE) has been reported in Japan (Yamauchi, 2002). It becomes a matter of great importance to solve the problems created by BSE. One alternative is to replace bovine collagen with another source. Until now, as part of a study looking at the effective use of underutilized resources, we have reported the preparation and characterization of collagens from aquatic organisms (Nagai *et al.*, 1999, 2000, 2001, 2002a, 2002b; Nagai and Suzuki, 2000a, 2000b, 2000c, 2002). As a result, a great amount of collagen could be obtained from many marine vertebrates and invertebrates (Nagai *et al.*, 1999, 2000, 2001, 2002a, 2002b; Nagai and Suzuki, 2000a, 2000b, 2000c, 2002).

Japanese consume a wide range of fish species, tunas, prawns, shellfishes, octopi, and squids. Particularly, they daily eat sliced raw fresh, sashimi. Among them, Japanese common squid, arrow squid, and long-finned squid are a special choice. Sashimi and sushi preparation require the removal of skin. The skin is treated as waste at home, in fish shops, and fish processing and refrigerating factories. Diamondback squid (*Thysanoteuthis rhombus*) is a big species with a mantle growing up to about 80 cm. It is called "Taru-ika" or Ō-tobi-ika" in Japan and has thick skins. If substantial amounts of collagen could be obtained from wastes, they would provide an alternative to bovine collagen in food, cosmetics, and biomedical materials. This paper describes the preparation and characterization of collagen from diamondback squid outer skin.

Materials and Methods

Sample

Diamondback squid (*Thysanoteuthis rhombus*) was caught in Shimonoseki City, Yamaguchi Prefecture, Japan. The outer skin was removed, washed with distilled water, lyophilized, and stored at – 85 °C until use.

Preparation of collagen sample

All preparative procedures were carried out at 4 °C. The lyophilized outer skin was treated with 0.1 M NaOH to remove noncollagenous proteins and pigments for 3 d by changing the solution once a day. Then the outer skin was washed with distilled water for 2 d by changing the solution, and

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the matter was extracted with 0.5 M acetic acid for 3 d. The extract was centrifuged at 50,000 × *g* for 1 h. The supernatants were pooled and salted out by adding NaCl to a final concentration of 0.8 M followed by precipitation of the collagen by addition of NaCl (final concentration of 2.3 M) at a neutral pH (in 0.05 M Tris-HCl, pH 7.5). The resultant precipitate was obtained by centrifugation at 50,000 × *g* for 1 h, and dissolved in 0.5 M acetic acid, dialyzed against 0.1 M acetic acid, distilled water, and then lyophilized (acid-solubilized collagen). On the other hand, the residue from the acetic acid extraction was washed with distilled water, suspended in 0.5 M acetic acid, and digested with 1% (w/v) pepsin (EC 3.4.23.1; 2 × crystallized; 3,085 U/mg protein; Sigma, USA) for 48 h. The viscous solution was centrifuged at 50,000 × *g* for 1 h, and the supernatants were pooled and dialyzed against 0.02 M Na_2HPO_4 (pH 7.2) for 3 d with the change of solution to inactivate the pepsin. After centrifugation at 50,000 × *g* for 1 h, the precipitate was dissolved in 0.5 M acetic acid and salted out by adding NaCl to a final concentration of 0.8 M, followed by precipitation of the collagen by addition to a final concentration of 2.3 M NaCl in 0.05 M Tris-HCl (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 (pepsin-solubilized collagen).

SDS-polyacrylamide gel electrophoresis (SDS-PAGE)

SDS-PAGE was performed as described previously (Nagai *et al.*, 2002b). After the electrophoresis, the gels were stained with Coomassie Brilliant Blue R-250 (Fluka Fine Chemical Co., Ltd., Tokyo, Japan) and destained with 5% methanol and 7.5% acetic acid.

Peptide mapping

The collagen samples (0.5 mg) were dissolved in 0.1 M sodium phosphate buffer (pH 7.2) containing 0.5% SDS and heated at 100 °C for 5 min. After cooling in ice, the digestion was done at 37 °C for 30 min using 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 mass fraction of 2%, the proteolysis was stopped by boiling for 5 min. SDS-PAGE was performed by the method of Laemmli (1970) using 15% gels. Molecular weight markers purchased from Sigma (USA) were used as marker proteins.

Subunit composition

To separate the subunits of collagen samples, the sample was applied to a CM-Toyopearl 650M (Tosoh Co., Tokyo, Japan) column chromatography. 20 mg of the collagen sample were dissolved in 20 mM sodium acetate buffer (pH 4.8) containing 6 M urea at 4 °C, denatured at 45 °C for 30 min, and the solution was centrifuged at 50,000 × *g* at 20 °C for 1 h. The supernatants were applied to a CM-Toyopearl 650M column (1.0 × 6.0 cm) previously equilibrated with the same buffer. Each subunit was eluted with a linear gradient of 0 to 0.15 M NaCl in the same buffer at a flow rate of 0.8 ml/min. The subunit components were detected in absorbance at 230 nm, and the fractions indicated by the numbers were examined by SDS-PAGE.

Denaturation temperature (T_d)

T_d was measured by the method of Nagai *et al.* (2002b). 5 ml of 0.03% collagen solution in 0.1 M acetic acid were used for viscosity measurements. T_d was determined as the temperature at that the change in viscosity was half completed using a Canon-Fenske type viscometer with an average shear gradient of 400 s^{-1}. Each point is the mean of triplicate determinations.

Amino acid composition

Collagen samples were hydrolyzed under reduced pressure in 6 M HCl at 110 °C for 24 h, and the hydrolysates were analyzed on a JASCO liquid-chromatography system by on-line precolumn derivatization with *o*-phthalaldehyde. This system consisted of a JASCO PU-2080 plus intelligent HPLC-pump, a JASCO FP-2020 plus intelligent fluorescence detector, a JASCO CO-2060 plus intelligent column thermostat, a JASCO DG-2083-53 3-line degasser, a JASCO LG-2080-02 ternary gradient unit, a JASCO AS-2057 plus intelligent sampler, and a JASCO CrestPak C18S (∅ 4.6 × 150 mm) reversed-phase column. The excitation and emission wavelengths were set at 345 and 455 nm, respectively. Eluents were filtered through Millipore membrane filters (pore size 0.45 μm).

Results and Discussion

The outer skins of diamondback squid were hardly solubilized using 0.5 M acetic acid. The yield of acid-solubilized collagen was very low, about 1.3% on a dry weight basis. On the contrary, pepsin-solubilized collagen was perfectly solubilized from the residue from the acetic acid extraction, and was effectively purified by differential salt precipitation. The yield of pepsin-solubilized collagen was very high, about 35.6% on a dry weight basis. This result was similar to that of fish skin [Japanese sea bass (51.4%), chub mackerel (49.8%), and bullhead shark (50.1%) (Nagai and Suzuki, 2000a); ocellate puffer fish (44.7%) (Nagai *et al.*, 2002a)], perple sea urchin test (35.0%) (Nagai and Suzuki, 2000b), fish bone [Japanese sea bass (40.7%), horse mackerel (43.5%), and ayu (53.6%) (Nagai and Suzuki, 2000c)], edible jellyfish exumbrella (46.4%) (Nagai *et al.*, 1999), rhizostomous jellyfish mesogloea (35.2%) (Nagai *et al.*, 2000), *C. arakawai* arm (62.9%) (Nagai *et al.*, 2002b), paper nautilus outer skin (50.0%) (Nagai and Suzuki, 2002), and cuttlefish outer skin (35.0%) (Nagai *et al.*, 2001). It suggests that a great amount of collagen can be obtained from aquatic animals. By using 3.5% SDS-PAGE, it was found that acid-solubilized collagen and pepsin-solubilized collagen comprised only one α chain, $\alpha 1$ (Fig. 1). Moreover, a small amount of β chain was obtained in these collagen samples. These collagens, however, were poor in inter- and intra-molecular crosslinked components. In electromobility, the positions of α chain of these collagens were similar to those of porcine skin $\alpha 1$ chain. On the other hand, the existence of $\alpha 2$ and $\alpha 3$ chains were not identified under the electrophoretic conditions.

To compare directly the pattern of peptide fragmentation with the porcine skin collagen, the denatured pepsin-solubilized collagen from diamondback squid outer skin and porcine collagen were examined by 15% SDS-PAGE. As a result, the electrophoretic pattern of pepsin-solubilized

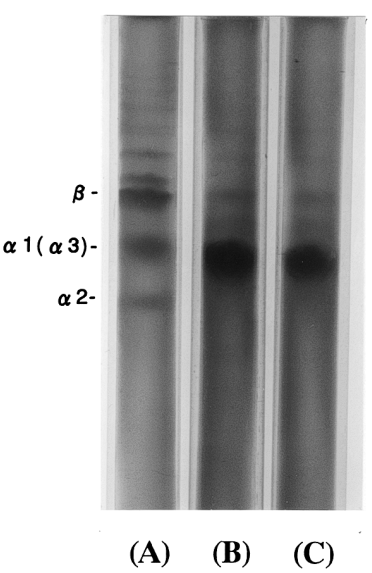

Fig. 1. SDS-polyacrylamide gel electrophoresis of porcine skin type I collagen and diamondback squid outer skin collagens on 3.5% gels containing 3.5 M urea. (A): Porcine collagen; (B): acid-solubilized collagen from diamondback squid outer skin; (C): pepsin-solubilized collagen from diamondback squid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

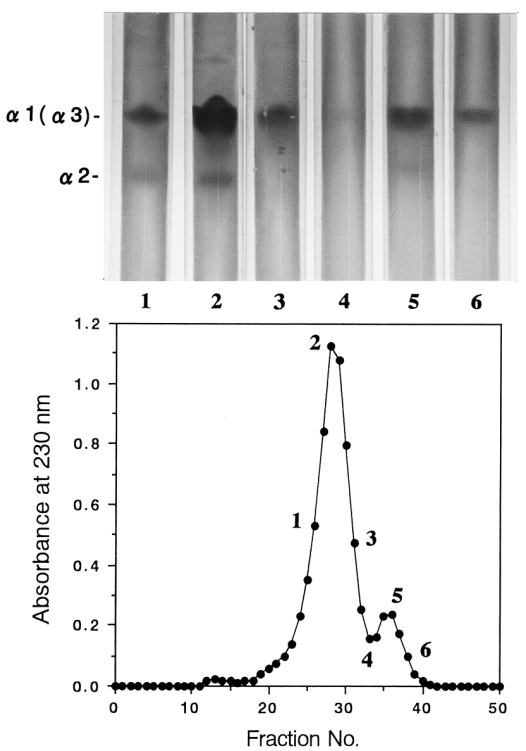

Fig. 2. CM-Toyopearl 650M column chromatography of denatured pepsin-solubilized collagen from diamondback squid outer skin. A 1.0 × 6.0 column of CM-Toyopearl 650M was equilibrated with 0.02 M sodium acetate buffer (pH 4.8) containing 6 M urea, and maintained at 37 °C. The collagen sample (20.0 mg) was dissolved in 5 ml of the same buffer, denatured at 45 °C for 30 min, and then eluted from the column with a linear gradient of 0 to 0.15 M NaCl at a flow rate of 0.8 ml/min. The fractions indicated by the numbers were examined by SDS-PAGE.

collagen from diamondback squid outer skin was different from that of porcine. It reveals that the peptide fragments of this collagen were fairly different from that of porcine skin.

To determine the subunit composition of pepsin-solubilized collagen from diamondback squid outer skin, the denatured collagen was applied to a CM-Toyopearl 650M column chromatograph. The chromatographic fractions were identified using SDS-PAGE. As shown in Fig. 2, it was separated into two fractions containing an α chain as a major component. The result suggests that this collagen consists of two α chains. That is, these chains were $\alpha 1$ (fraction numbers 26 to 31), $\alpha 2$ (fraction numbers 26 and 28), and $\alpha 3$ (fraction numbers 36 and 38) in the order of their elution positions. To confirm each chain, several fractions as indicated by the numbers were analyzed by SDS-PAGE. It was found that this collagen had a chain composition of $\alpha 1 \alpha 2 \alpha 3$ heterotrimer, different from those of other squids: cuttlefish, $(\alpha 1)_2 \alpha 2$ heterotrimer (Nagai *et al.*, 2001), and *Todarodes pacificus*, $(\alpha 1)_2 \alpha 2$ heterotrimer (Mizuta *et al.*, 1994a, 1994b).

T_d of pepsin-solubilized collagen from diamondback squid outer skin was calculated from the thermal denaturation curves. For comparison, T_d of porcine skin collagen was measured under the same conditions. Diamondback squid outer skin had a T_d of about 27.5 °C which was lower by about 10 °C than that of porcine skin collagen (T_d = 37.0 °C). This value was the same as obtained from other aquatic animals (T_d = 16.8–31.7 °C) (Nagai *et al.*, 1999, 2000, 2001, 2002a, 2002b; Nagai and Suzuki, 2000a, 2000b, 2000c, 2002). It suggests that the stability of collagen was correlated with body temperature and environmental temperature as Rigby (1968) reports.

Amino acid	
Hydroxyproline	89
Aspartic acid	65
Threonine	28
Serine	47
Glutamic acid	94
Proline	97
Glycine	320
Alanine	83
Half-cystine	0
Valine	24
Methionine	1
Isoleucine	21
Leucine	29
Tyrosine	5
Phenylalanine	10
Tryptophan	0
Lysine	13
Histidine	18
Arginine	56
Total	1000

Table I. Amino acid composition of diamondback squid outer skin pepsin-solubilized collagen, residues/1000.

Amino acid composition was investigated in pepsin-solubilized collagen from diamondback squid outer skin and was expressed as residues per 1000 total residues. As a result, amino acid composition of this collagen was similar to that from cuttlefish outer skin (Nagai *et al.*, 2001) (Table I). Glycine (320 residues) was the most abundant amino acid and accounted for more than 30% of all amino acids in this collagen. Proline (97 residues), alanine (83 residues), and glutamic acid (94 residues) were present in relatively high amount. Hydroxyproline is considered as an indicator in the stability of collagen sample. It showed approximately 89 residues and the degree of hydroxylation of proline was calculated to be 47.8%.

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