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Isolation and characterisation of collagen from the outer skin waste material of cuttlefish (*Sepia lycidas*)

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Abstract

In an investigation into making more effective use of underutilized resources, collagen was prepared from the outer skin of cuttlefish (*Sepia lycidas*). Initial extraction of the cuttlefish outer skin in acetic acid yielded only 2% of collagen (dry weight basis). On subsequent digestion of the residue with 10% pepsin (w/v), a solubilized collagen (PSC) was obtained in a yield of 35% (dry weight basis). With respect to PSC, it had a chain composition of $(\alpha 1)_2\alpha 2$ heterotrimer similar to Japanese common squid. Moreover, the denaturation temperature of this collagen was 27°C which is about 10°C lower than that of porcine collagen. This report indicates that cuttlefish waste materials have potential in supplementing the skin of land vertebrates as a source of collagen.

Keywords: Isolation; Characterisation; Cuttlefish

1. Introduction

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 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.

Japanese consume a wide range of fish species, tunas, prawns, squids daily and in particular, a sliced raw fresh, *sashimi*. Among these, squids such as *Sepia lycidas* are an especial choice. *Sashimi* and *sushi* preparation requires the removal of skin. In particular, cuttlefish have thick skins, but these are treated as wastes in the home, fish shops, fish processing and refrigerated factories. If these wastes were dumped as 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 & Suzuki, 2000a,b,c; Nagai et al., 1999, 2000). This paper aims to isolate and characterize the collagen from the outer skin of cuttlefish (*S. lycidas*).

2. Materials and methods

2.1. Sample

Cuttlefish, *S. lycidas*, were obtained from Itano Refrigerated Food Co., Ltd. Tokushima Factory, Japan. The outer skins were removed, cut into small pieces and stored at −85°C until used.

2.2. Preparation of collagen from the outer skin

All the preparative procedures were performed at 4°C. The skins were extracted with 0.1 M NaOH to remove noncollagenous proteins for 3 days, then washed with distilled water and lyophilized. The lyophilized

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skins were extracted with 0.5 M acetic acid for 3 days, and the extracts were centrifuged at 50,000×*g* for 1 h. The residue was re-extracted with the same solution for 2 days, and the extract was centrifuged under the same conditions. Each solution was mixed and salted out by adding NaC1 to a final concentration of 0.8 M and followed by precipitation of the collagen by the addition of NaC1 (final concentration of 2.3 M) at a neutral pH (in 0.05 M Tris–HC1, 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, and distilled water and then lyophilized (ASC).

The residue from the acetic acid extraction was suspended in 0.5 M acetic acid and was digested with 10% (w/v) pepsin (EC 3. 4. 23. 1; 2×crystallized, Sigma, USA) at 4°C for 48 h. The pepsin-solubilized collagen was centrifuged at 50,000×*g* for 1 h and the supernatants were dialyzed against 0.02 M Na_2HPO_4 (pH 7.2) for 3 days with change of solution once per 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 0.8 M and followed by precipitation of collagen by further addition, to a 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 and dissolved in 0.5 M acetic acid, dialyzed against 0.1 M acetic acid, distilled water and then lyophilized.

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

SDS-PAGE was performed as described previously (Nagai et al., 1999). The collagen sample was dissolved in 0.02 M sodium phosphate (pH 7.2) containing 1% SDS and 3.5 M urea. Electrophoresis was performed on 3.5% gels in 0.1 M sodium phosphate buffer (pH 7.2) containing 0.1% SDS. After electrophoresis, gels were visualized with Coomassie Brilliant Blue R-250.

2.4. Peptide mapping

A collagen sample (0.2 mg) was dissolved in 0.1 M sodium phosphate buffer (pH 7.2) containing 0.5% SDS, 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* (Wako Pure Chemicals, 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 15% gel.

2.5. CM-Toyopearl 650M column chromatography

The subunit components of this collagen were separated by using a CM-Toyopearl 650M (Tosoh Co., Japan) column chromatograph. The collagen sample (10.0 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 20°C 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 NaCl in the same buffer at a flow rate of 0.8 ml/min. Absorbance at 230 nm was used to monitor the column chromatography.

2.6. Determination of denaturation temperature

The denaturation temperature (Td) was measured by the method of Nagai et al. (1999). Five millilitres of 0.03% collagen solution in 0.1 M acetic acid was used for viscosity measurements. Its measurement was done by 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 min. The T_d was determined as the temperature at which the change in viscosity was half completed. Each point is the mean of triplicate determinations.

2.7. Amino acid composition

A collagen sample was hydrolyzed under reduced pressure in 6 M HC1 at 110°C for 24 h, and the hydrolysates were analyzed on a Nihon Electronics Co. amino acid analyzer (JLC-300).

3. Results and discussion

3.1. Isolation of cuttlefish collagen

The cuttlefish outer skins were hardly solubilized with 0.5 M acetic acid. The yield of ASC was very low and its value was about 2.0% on the basis of lyophilized dry weight. On the other hand, by treating the residue with 10% (w/v) pepsin in 0.5 M acetic acid, PSC was perfectly solubilized. PSC was precipitated with 0.8 M NaCl in acid solution and followed by the addition of 2.3 M NaCl at neutral pH. PSC obtained was a pinkish fibre, and it was supposed that a pigment, such as ommochrome, remained in this collagen sample. The yield of PSC was very high and its value was about 35% on the basis of lyophilized dry weight. When these collagens were examined by 3.5% SDS-PAGE, ASC showed only a single α band and it seemed that this was α 1 (data not shown). PSC was shown to comprise two α chains, α 1 and α 2 (Fig. 1). Moreover, a great amount of β chain was obtained in PSC. These collagens,

however, were poor in inter- and intra-molecular cross-linked components.

3.2. Peptide mapping

The collagens digested by lysyl endopeptidase were examined by SDS-PAGE to easily compare the pattern of peptide fragment with porcine skin collagen. As a result, the electrophoretic pattern of PSC was similar to that of ASC (Fig. 2). Moreover, these patterns were also similar to that of porcine material. The pattern of the peptide fragment of cuttlefish outer skin collagen may be closely similar to that of porcine skin.

3.3. Subunit composition

The denatured pepsin-solubilized collagen was applied to a CM-Toyopearl 650M column chromatograph. As a result, a large peak was resolved having a shoulder on the right and it was supposed that the second and third fractions contain α chains as major components (Fig. 3). To identify each α chain, several chromatographic fractions as indicated by the number, were analyzed by SDS-PAGE. Judging from the electrophoretic pattern, it was found that this collagen had a chain composition of $(\alpha\,1)_2\alpha 2$ heterotrimer, similar to those from other squids (Mizuta, Yoshinaka, Sato, Itoh & Sakaguchi, 1994; Mizuta, Yoshinaka, Sato & Sakaguchi, 1994; Shadwick, 1985).

3.4. Denaturation temperature

Td of this collagen sample was calculated from the thermal denaturation curves. In comparison, T_d of porcine skin collagen was measured under the same conditions. As shown in Fig. 4, PSC had a T_d of 27.0°C that was lower by about 10°C, than that of porcine skin collagen (37.0°C). This value was the same as obtained from other marine organisms: Alaska pollack skin (16.8°C) and swim bladder (18.4°C; Kimura & Ohno, 1987); muscle of carp (32.5°C), eel (30.2°C), common mackerel (26.9°C), saury (24.0°C), chum salmon (20.6°C) and skin of carp (31.7°C), eel (29.3°C), common mackerel (26.1°C), saury (23.0°C), chum salmon (19.4°C; Kimura, Zhu, Matsui, Shijoh & Takamizawa, 1988); body wall of starfish (23.0°C; Kimura, Omura, Ishida & Shirai, 1993); edible jellyfish exumbrella (26.0°C.) (Nagai et al., 1999); skin of Japanese sea bass (26.5°C), chub mackerel (25.6°C), bullhead shark (25.0°C), bone of Japanese sea bass (30.0°C), skipjack and ayu (29.7°C), yellow sea bream and horse mackerel (29.5°C) and Japanese sea bass fin (29.1°C; Nagai & Suzuki, 2000a); rhizostomous jellyfish mesogloea (28.8°C; Nagai et al., 2000); purple sea urchin test (28.0°C; Nagai & Suzuki, 2000b). It is known that the stability of collagen is correlated with environmental and body temperature (Rigby, 1968). This result well supports these findings.

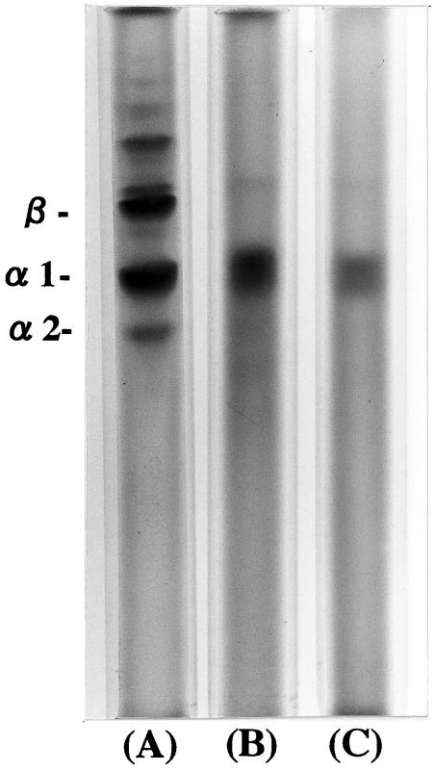

Fig. 1. SDS-polyacrylamide gel electrophoresis of porcine skin type I collagen and cuttlefish outer skin collagen on 3.5% gels containing 3.5 M urea: (A) porcine collagen, (B) cuttlefish acid-soluble collagen, (C) cuttlefish pepsin-solubilized collagen.

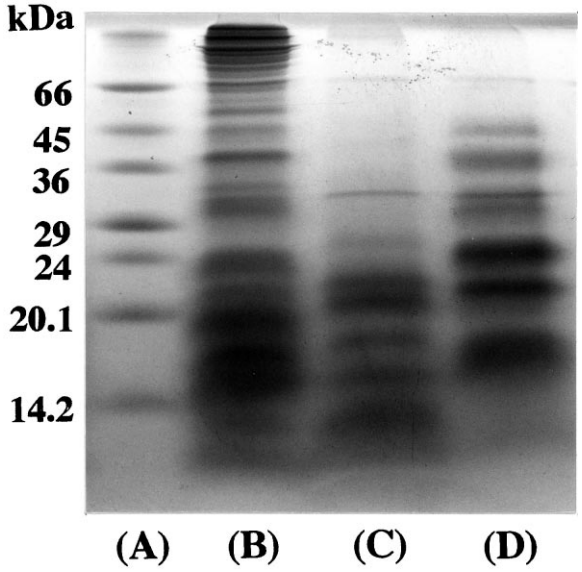

Fig. 2. Peptide mapping of lysyl endopeptidase digests from cuttlefish outer skin collagen. (A): molecular weight marker, (B) porcine collagen, (C) cuttlefish acid-soluble collagen, (D) cuttlefish pepsin-solubilized collagen.

3.5. Amino acid composition

The amino acid composition, expressed as residues per 1000 total residues, is shown in Table 1. This shows that glycine was the most abundant amino acid in cuttlefish skin collagen and that there were relatively high contents of proline, hydroxyproline and glutamic acid, decreasing in that order. The value of glycine was approximately 318 residues. The degree of hydroxylation of proline was calculated to be 47.9%.

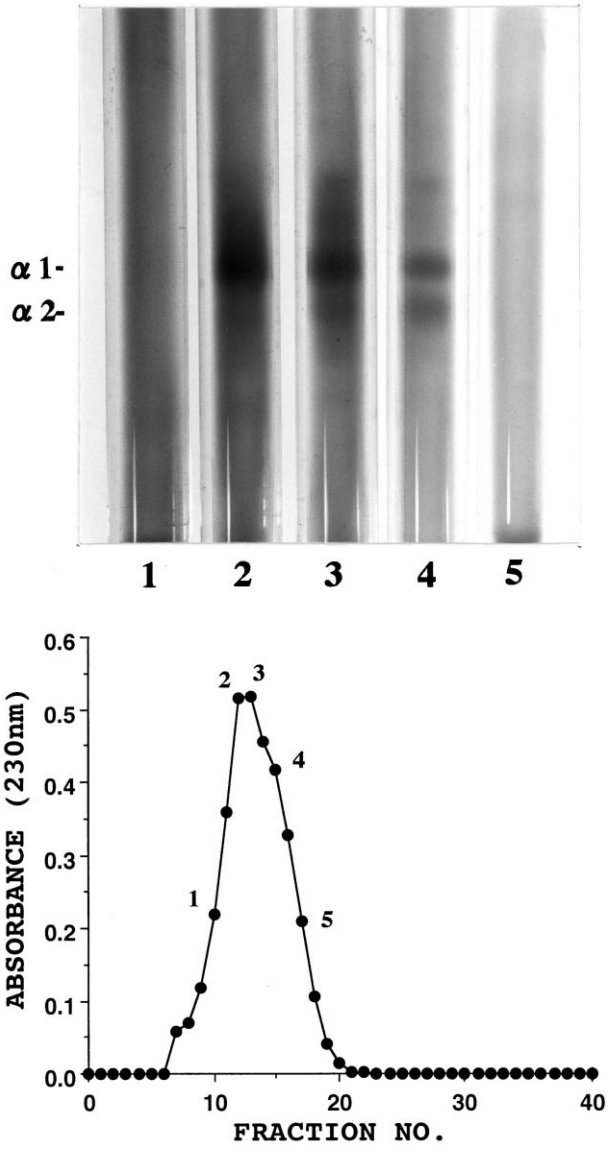

Fig. 3. CM-Toyopearl 650 M column chromatography of denatured cuttlefish outer skin collagen. A 1.0×5.0 cm column of CM-Toyopearl 650 M was equilibrated with 20 mM sodium acetate buffer (pH 4.8) containing 6 M urea, and maintained at 37°C. The collagen sample (20 mg) was dissolved in 5 ml of the same buffer, denatured at 45°C for 30 min, and then eluted from the column with a linear gradient of 0–0.15 M NaCl at a flow rate of 0.8 ml/min. The fractions indicated by the numbers were examined by SDS-PAGE.

In general, it is well-known that the extractive content of squids is the same as red-meat fish and squid is richer in betaine, taurine and proline than fish. Although the nutritional values of squid are fairly high, these useful resources, particularly cuttlefish outer skin, may be wasted, except that some waste is used in fish meal manufacture. In this study, it was found that a large amount of collagen can be obtained from cuttlefish outer skin. This result indicates that cuttlefish outer skins have potential as alternative sources of

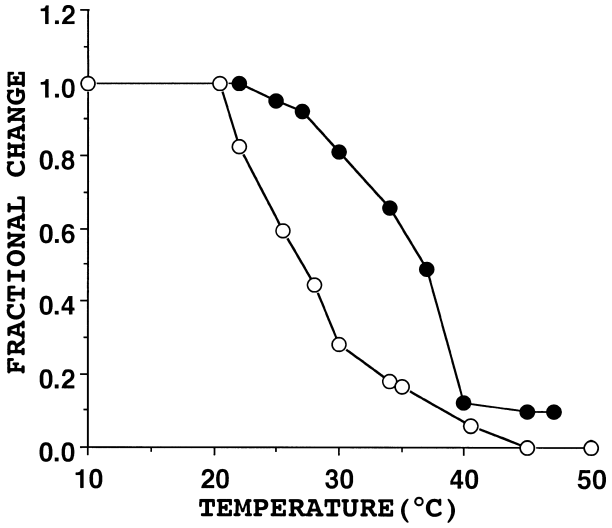

Fig. 4. Thermal denaturation curve of cuttlefish outer skin collagen solution as measured by viscosity in 0.1 M acetic acid. The incubation time at each temperature was 30 min. Collagen concentration 0.03%, ● porcine skin collagen; ○ cuttlefish outer skin collagen.

Table 1
Amino acid composition of cuttlefish outer skin pepsin-solubilized collagen, residues/1000

Amino acid	
Hydroxyproline	90
Aspartic acid	64
Threonine	29
Serine	48
Glutamic acid	92
Proline	98
Glycine	318
Alanine	83
Half-cystine	2
Valine	23
Methionine	1
Isoleucine	21
Leucine	29
Tyrosine	5
Phenylalanine	10
Tryptophan	0
Lysine	13
Histidine	18
Arginine	56
Total	1000

collagen if we can improve the thermal stability of this collagen.

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