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Collagen of the skin of ocellate puffer fish (*Takifugu rubripes*)

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Abstract

Collagens (acid-solubilized and pepsin-solubilized collagens) were prepared from ocellate puffer fish skin and partially characterized. With respect to the pepsin-solubilized collagen, it was a heterotrimer with a chain composition of $(\alpha 1)_2\alpha 2$. The patterns of peptide fragments were different from skin collagens of other species. The denaturation temperature was 28 °C, about 9 °C lower than that of porcine skin collagen. On the other hand, the yields of acid-solubilized and pepsin-solubilized collagens were very high, 10.7% and 44.7%, respectively, on a dry weight basis. These results suggest that ocellate puffer fish skin has potential as an alternative source of collagen for use in various fields.

Keywords: Collagen; Ocellate puffer fish; Yield; Underutilized resources

1. Introduction

Edible puffer fish, such as *Takifugu rubripes*, *Takifugu porphyreus*, and *Takifugu vermicularis*, belong to the order Tetraodontidae. It is known that the ovary and liver of many puffer fish contain a toxin, tetrodotoxin, one of the most potent non-protein neurotoxins. Pufferfish of the genus *Takifugu* are mainly distributed in the East China Sea and its surrounding waters, in which about 23 species have been recognized (Masuda, Takahashi, Dotsu, Miyaki, Tabeta, & Matsuura, 1986). Among them, *T. rubripes* is treated as a fish of the highest quality in Japan. It is covered with thick skin, and its skin is non-toxic. The Japanese eat the skin as a garnishing of sliced raw fish, *sashimi*, and as a vinegared dish. Moreover, the Japanese eat its muscle as *sashimi*, a pot of fish and vegetables cooked before the dinners *chiri-nabe*, and *miso* soup (Japan Association of Training Colleges for Cooks, 1996).

Collagen is the predominant protein in the living body. The main sources of industrial collagen are limited to those from pig and bovine skin and bones. It is known that the skin of puffer fish contains a large quantity of collagen, and that skin of *T. rubripes* has potential as an important source of collagen. In this paper, the preparation and characterisation of collagen from the skin of *T. rubripes* are described.

2. Materials and methods

2.1. Fish

Ocellate puffer fish, *T. rubripes*, were purchased from the local wholesale market in Shimonoseki City, Yamaguchi Prefecture, Japan. The skins were removed, cut into small pieces, and stored at −25 °C until required.

2.2. Preparation of skin collagen

The collagen was prepared by the method of Nagai and Suzuki (2000a). All the preparative procedures were performed at 4 °C. The skins were treated with 0.1 N NaOH to remove noncollagenous proteins and pigments, then washed with distilled water, and lyophilized. To

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remove fat in the lyophilized powder, it was treated with 10% butyl alcohol for 2 days once per day, washed with distilled water, and lyophilized. The matter was extracted with 0.5 M acetic acid for 3 days, and the extract was centrifuged at 20,000×*g* for 1 h. The supernatants were salted-out by adding NaCl to a final concentration of 0.7 M and followed by precipitation of the collagen by the addition of NaCl (final concentration of 2.3 M) in 0.05 M Tris–HCl (pH 7.5). The resultant precipitate was separated by centrifugation at 20,000×*g* for 1 h, and dissolved in 0.5 M acetic acid. The solution obtained was dialyzed against 0.1 M acetic acid solution, distilled water, and then lyophilized (acid-solubilized collagen; ASC). The residue was suspended in 0.5 M acetic acid and digested with 10% (w/v) pepsin (EC 3. 4. 23. 1; 2×crystallized; 3085 U/mg protein, Sigma, USA) for 48 h at 4 °C. The viscous solution was centrifuged at 20,000×*g* for 1 h and the supernatants were dialyzed against 0.02 M Na_2HPO_4 (pH 7.2) for 3 days with a change of solution once per day. After the dialysate was centrifuged at 20,000×*g* for 1 h, the precipitate was dissolved in 0.5 M acetic acid and was salted-out by adding NaCl to a final concentration of 0.7 M and followed by precipitation by addition of NaCl (final concentration of 2.2 M) in 0.05 M Tris–HCl (pH 7.5). The resultant precipitate was separated by centrifugation at 20,000×*g* for 1 h, dissolved in 0.5 M acetic acid, dialyzed against the same solution, and then lyophilized (pepsin-solubilized collagen; PSC).

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

SDS-PAGE was performed as previously described (Nagai & Suzuki, 2000c). After 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. Subunit composition

To separate the subunit components of this collagen, the collagen sample was applied to a CM-Toyopearl 650M (Tosoh Co. Tokyo, Japan) column. Briefly, 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 centrifuged at 20,000×*g* at 20 °C for 1 h. The supernatants were applied to a CM-Toyopearl 650M column (1.0×5.0 cm), previously equilibrated with the same buffer. Each subunit was eluted with a linear gradient of 0–0.15 M NaCl in the same buffer at a flow rate of 0.8 ml/min. The subunit components were detected by absorbance at 230 nm. The fractions indicated by the numbers were examined by SDS-PAGE.

2.5. Peptide mapping

The collagen sample was digested by lysyl endopeptidase from *Achromobacter lyticus* (EC 3. 4. 21. 50; 4.5 amidase activity/mg protein; Wako Pure Chemicals, Osaka, Japan), applied to 15% gel. SDS-PAGE was performed by the method of Laemmli (1970).

2.6. Denaturation temperature

The denaturation temperature (T_d) was measured by the method of Nagai et al. (2000). T_d was determined as the temperature at which the change in viscosity, using a Canon-Fenske type viscometer with an average shear gradient of 400 s^{-1}, was half completed.

2.7. Amino acid composition

A collagen sample was hydrolyzed under reduced pressure in 6 M HCl at 110°C for 24 h, and the hydrolysates were analyzed on a Shimadzu amino acid analyzer (LC-10A).

3. Results and discussion

The skin of ocellate puffer fish was not completely solubilized with 0.5 M acetic acid. In this way it was similar to the skin of *Callistoctopus arakawai* arm (Nagai, Nagamori, Yamashita, & Suzuki, 2001), cuttlefish outer skin (Nagai, Yamashita, Taniguchi, Kanamori, & Suzuki, 2001) and paper nautilus outer skin (Nagai & Suzuki, 2001). This result was different from those for Japanese sea bass, chub mackerel, and bullhead shark skin (Nagai & Suzuki, 2000a). The collagen of ocellate puffer fish skin was easiliy solubilized by limited pepsin proteolysis. This was similar to those of edible jellyfish exumbrella (Nagai et al., 1999) and rhizostomous jellyfish mesogloea (Nagai et al., 2000). PSC was effectively purified by differential salt precipitation. The yield of PSC was very high (44.7% on a dry weight basis). On the other hand, the yield of ASC was low (10.7% on a dry weight basis). This result was similar to those for fish skin (Japanese sea bass, 51.4%, chub mackerel, 49.8%, and bullhead shark, 50.1%) (Nagai & Suzuki, 2000a), purple sea urchin test (35.0%) (Nagai & Suzuki, 2000b), fish bone (Japanese sea bass, 40.7%, horse mackerel, 43.5%, and ayu, 53.6%) (Nagai & 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, Nagamori, Yamashita, & Suzuki et al., 2001), cuttlefish outer skin (35.0%) (Nagai, Yamashita, Taniguchi, Kanamori, & Suzuki, 2001), and paper nautilus outer skin (50.0%) (Nagai & Suzuki, 2001). It appears that a large amount of collagen can be obtained from aquatic animals. The collagen obtained was

examined by SDS-PAGE using 3.5% gel. It was found that these collagens comprised at least two different α chains;α 1 and α 2 (Fig. 1). In electrophoretic mobility, the positions of α chains of these collagens differed from those of porcine skin α chains. That is, α chains of ocellate puffer fish skin collagen are distinct in this primary structure. If other α chains, such as α3 and α4, were present in these collagens, they were not separated from the corresponding α 1 chain under the electrophoretic conditions employed.

To determine the subunit composition of ocellate puffer fish skin PSC, the denatured collagen was further resolved by CM-Toyopearl 650M column chromatography and its chromatographic fractions were identified by SDS-PAGE. It was shown that PSC consists of two α chains (Fig. 2). This collagen is a heterotrimer with a chain composition of $(\alpha 1)_2\alpha 2$. Kimura, Ohno, Miyauchi, and Uchida (1987) examined the fish skin collagens and reported that the α 3 chain was widely distributed in teleosts, such as eel, sardine, chum salmon, rainbow trout, carp, anger, Alaska pollack, cod, halfbeak, common mackerel, tilapia, red barracuda, northern dab, and file fish. In the previous paper (Kimura, 1985; Kimura & Ohno, 1987; Kimura et al., 1987; Piez, 1965), it was reported that the α 3 chain was detected in 14 fish species of 17 teleosts. Moreover, we have reported the existence of α 3 chain in edible jellyfish exumbrella (Nagai et al., 1999), rhizostomous jellyfish mesogloea (Nagai et al., 2000), ayu bone (Nagai & Suzuki, 2000c), paper nautilus outer skin (Nagai & Suzuki, 2001), and *C. arakawai* arm (Nagai, Nagamori, Yamashita, & Suzuki, 2001a). Now our present data are different from those of previous papers.

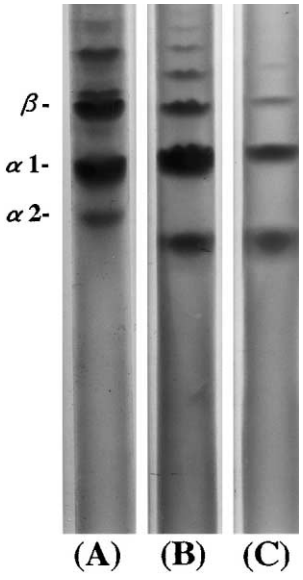

Fig. 1. SDS-polyacrylamide gel electrophoresis of porcine skin type I collagen and ocellate puffer fish skin collagens on 3.5% gels containing 3.5 M urea. (A) Porcine; (B) ocellate puffer fish acid-solubilized collagen; (C) ocellate puffer fish pepsin-solubilized collagen.

The collagens digested by lysyl endopeptidase were applied to 15% gel SDS-PAGE to compare the patterns of peptide fragment with porcine and other fish species skin collagens. As a result, the electrophoretic pattern of ocellate puffer PSC was similar to that of ASC (Fig. 3).

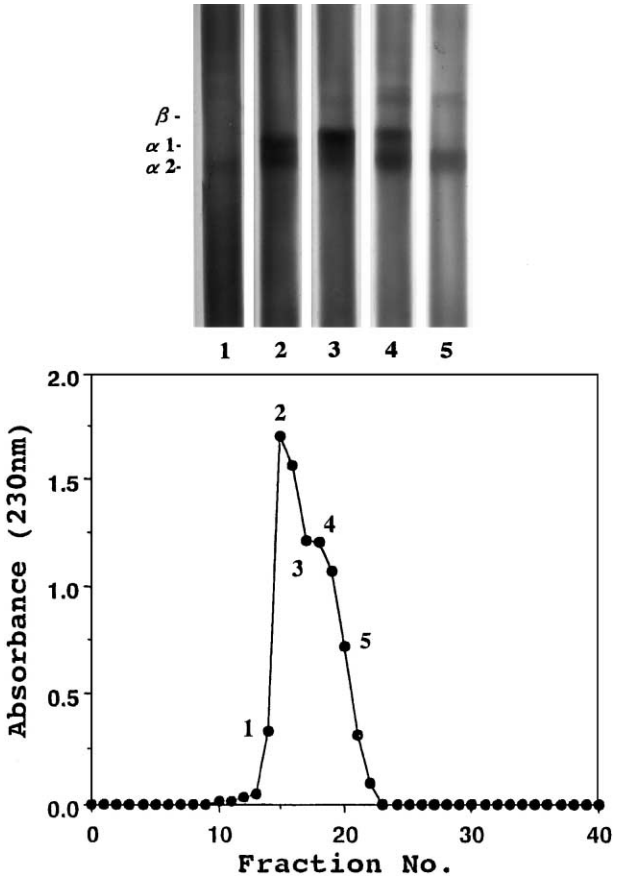

Fig. 2. CM-Toyopearl 650M column chromatography of denatured ocellate puffer fish skin collagens. The fractions indicated by the numbers were examined by SDS-PAGE.

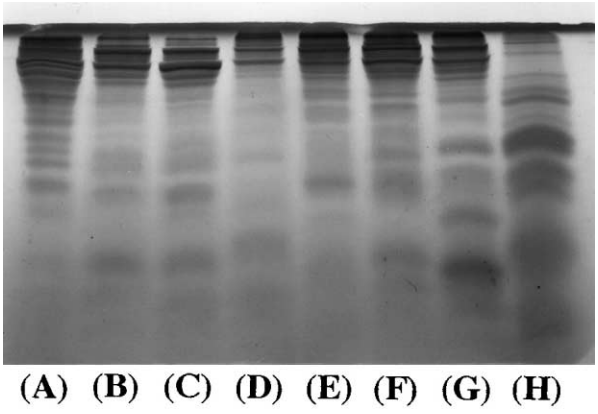

Fig. 3. Comparison, by peptide mapping, of lysyl endopeptidase digests from several fish skin collagens. (A) Porcine collagen; (B) ocellate puffer fish acid-solubilized collagen; (C) ocellate puffer fish pepsin-solubilized collagen; (D) chub mackerel collagen; (E) Japanese sea bass collagen; (F) yellowtail collagen; (G) bullhead shark collagen; and (H) ayu collagen.

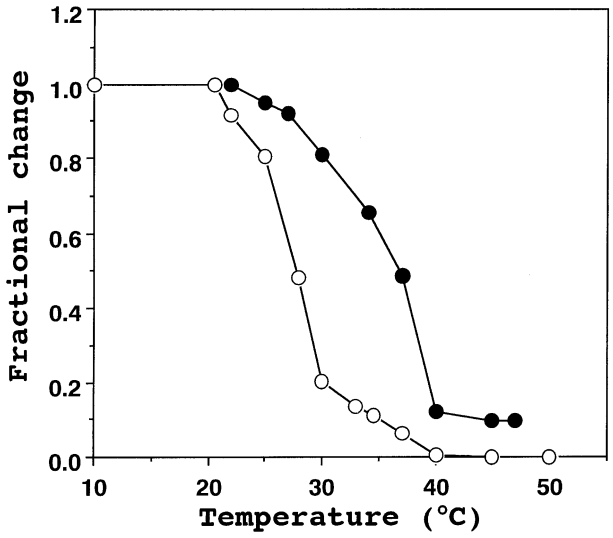

Fig. 4. Thermal denaturation curve of ocellate puffer fish skin collagen solution as measured by viscosity in 0.1 M acetic acid. The incubation time at each temperature was 30 min. Collagen concentration: 0.03%; (●): porcine skin collagen; (○): ocellate puffer fish skin collagen.

Table 1
Amino acid composition of ocellate puffer fish skin pepsin-solubilized collagen, residues/1000

Amino acid	
Hydroxyproline	67
Aspartic acid	50
Threonine	25
Serine	48
Glutamic acid	87
Proline	103
Glycine	351
Alanine	106
Half-cystine	2
Valine	17
Methionine	14
Isoleucine	12
Leucine	23
Tyrosine	4
Phenylalanine	10
Tryptophan	0
Lysine	19
Histidine	8
Arginine	54
Total	1000

Although the patterns of high molecular weight fragments were similar to each other, the patterns of these collagens were different from other species. It appears that the primary structure of collagen differs among species of teleosts.

The T_d of ocellate puffer PSC was calculated from the thermal denaturation curve. For comparison, T_d of porcine skin collagen was similarly measured. It was calculated that T_d of ocellate puffer PSC was about 28 °C (Fig. 4). This was about 9 °C lower than that of porcine skin collagen. This value was similar to those obtained from other marine organisms: Alaska pollack skin (16.8 °C) and swim bladder (18.4 °C) (Kimura & Ohno, 1987), muscles of carp (32.5 °C), eel (30.2 °C), common mackerel (26.9 °C), saury (24.0 °C), chum salmon (20.6 °C) and skins 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), *C. arakawai* arm (28.0 °C) (Nagai, Nagamori, Yamashita, & Suzuki, 2001a), (cuttlefish outer skin (27.0 °C) (Nagai, Yamashita, Taniguchi, Kanamori, & Suzuki, 2001b); and paper nautilus outer skin (27.0 °C) (Nagai & Suzuki, 2001). It has been suggested that the tendency for T_ds of marine organism to be lower than those of land animals is correlated with their environmental and body temperatures (Rigby, 1968).

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 ocellate puffer skin collagen and that there were relatively high contents of alanine, proline and glutamic acid, decreasing in that order. Glycine accounted for more than 30% of all amino acids in this collagen. Its value was approximately 351 residues. The degree of hydroxylation of proline was calculated to be 39.4%. In our previous paper, it was reported that the degrees of hydroxylation of proline were as follows: 32.8% (edible jellyfish exumbrella: Nagai et al., 1999), 48.0% (purple sea urchin test: Nagai & Suzuki, 2000b), 51.7% (*C. arakawai* arm: Nagai, Nagamori, Yamashita, & Suzuki, 2001a), and 47.9% (cuttlefish outer skin: Nagai, Yamashita, Taniguchi, Kanamori, & Suzuki, 2001). Among them, it was found that ocellate puffer skin collagen was unstable against temperature.

In conclusion, a great quantity of collagen could be obtained from ocellate puffer skin. This pufferfish is the bigest among about 23 puffer species that have been recognized in the East China Sea and its surrounding waters, and its skin is non-toxic like those of *T. rubripes*, *T. xanthopterus*, *Lagocephalus laevigatus inermis*, *Lagocephalus wheeleri*, and *Liosaccus pachygaster*. For these reasons, ocellate puffer skin has potential as an alternative source of collagen to cattle and porcine skin and bone. This study is only one useful report of many studies into making more effective use of underutilized resources such as collagen.

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