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Biochemical and immunochemical detection of types I and V collagens in tiger puffer *Takifugu rubripes*

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Abstract

Crude collagen fractions, prepared by alkali extraction from several parts (muscle, liver, swim bladder, bone, fin, gill, alimentary tract) of tiger puffer *Takifugu rubripes*, were analysed by biochemical and immunochemical techniques to examine the distribution of collagen types (types I and V collagens) among these parts. Collagens from these parts showed quite similar SDS–PAGE patterns and similar peptide maps. Immunoblot analyses were performed for these collagens using antisera against types I and V collagens isolated from pepsin-solubilized collagen of skin, revealing the existence of the positive components for these antisera in all of them. The results suggest that types I and V collagens may be widely distributed in these parts of tiger puffer.

Keywords: Collagen; Types; Fish; Tiger puffer; Muscle; Skin; Connective tissue

1. Introduction

Collagen, one of the extracellular matrix constituents of multicellular animals, not only plays important roles in the development of meat texture but has been utilized as a material for foods, cosmetics, pharmaceuticals and experimental reagents. The target of utilization has mainly been collagens derived from mammals such as cows and pigs. Aquatic animals, however, have been of increasing attention as a backup collagen resource since bovine spongiform encephalopathy (BSE) occurred and damaged various cattle industries.

Type I collagen is a major molecular species of fish, being widely distributed in their organs (Kimura, 2002) and is also a target collagen type for utilization (Nagai & Suzuki, 2000a, 2000b; Nomura, Sakai, Ishii, & Shirai, 1996). Fish muscles, however, also contain a minor molecular species, type V collagen, which has been revealed to be an important factor in *post-mortem* tenderization of raw meat (Sato et al., 1997, Sato, Koike, Yoshinaka, Sato, & Shimizu, 1994, 1991). Type V collagen or its constituent α chains have been reported to show distinctly different physiological functions from type I collagen, such as inhibitory effects on adhesion or proliferation of endothelial cells (Sato, Taira, Takayama, Ohtsuki, & Kawabata, 1995; Underwood, Bean, & Whitelock, 1998), on the epithelial cell cycle (Parekh, Wang, Makri-Werzen, Greenspan, & Newman, 1998) and on the attachment, spread and growth of smooth muscle cells (Sakata, Jimi, Takebayashi, & Marques, 1992, 1994). There seems to be a possibility of bringing fish type V collagen to effective use as a material for experimental reagents or pharmaceuticals, although it has been reported that the content of type V collagen is much lower than that of type I collagen (Sato et al., 1997, 1994, 1991).

Tiger puffer *Takifugu rubripes* is one of the commercially important fish species, being harvested wild or cultured in Japan. In addition, tiger puffer is regarded as biologically important species, for which a genome database has recently been established. As for collagen in tiger puffer, types I and V collagens were isolated from the pepsin-solubilized collagen preparations of muscle and skin (Sato et al., 1997; Yata, Yoshida, Fujisawa, Mizuta, & Yoshinaka, 2001). The report, however, is quite limited concerning the distribution of these

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collagen types in other parts of the fish. In the present study, the distribution of these collagen types was examined, in several parts of tiger puffer, by biochemical and immunochemical techniques to obtain fundamental information concerning textural development and industrial utilization of various parts of the fish.

2. Materials and methods

2.1. Materials

Tiger puffers *T. rubripes* (body weight, 120–200 g), reared at the Research Center for Marine Bioresources, Faculty of Biotechnology, Fukui Prefectural University (Obama City), were obtained alive. Eight parts (skin, muscle, liver, swim bladder, gill, fin, bone, alimentary tract) were dissected out from the bodies and stored at −80 °C until used.

2.2. Preparation of collagens

All procedures were performed in a cold room at 5 °C. Each part was cleaned of adhering tissues and materials and cut into small pieces. Soft parts (muscle, skin, liver, swim bladder and alimentary tract) were homogenized with ten volumes (v/w) of 0.1 M NaOH and extracted for 24 h. The extraction was done to remove non-collagenous proteins and to prevent the effects of endogenous proteases on collagen as described previously (Yoshinaka et al., 1985). The residue, after alkali extraction (RS-AL) was washed thoroughly with distilled water. Hard parts (bone, fin and gill) were cleaned of adhering tissues and decalcified with 0.5 M ethylene diamine tetraacetic acid (EDTA) solution, whose pH was adjusted to 8.0 with NaOH. The decalcified samples were homogenized as described above and the resultant residues (RS-AL) were washed thoroughly with distilled water. A small part of the RS-AL from the skin was lyophilized and the remainder was used for the consequent fractionation of collagen types. The RS-ALs from the other parts were all lyophilized.

2.3. Preparation and fractionation of pepsin-solubilized collagen

The RS-AL from the skin was digested with porcine pepsin (EC 3.4.23.1; Sigma, USA; crystallized and lyophilized) in 0.5 M acetic acid at an enzyme/substrate ratio of 1:20–1:50 (w/w) for 48 h at 5 °C. After centrifugation at 10,000*g* for 20 min, the collagen in the supernatant was used as a pepsin-solubilized collagen (PSC) preparation. The PSC was salted out by adding NaCl to give a final concentration of 2.0 M, and extracted with 0.5 M acetic acid containing 11.0% (w/v) ammonium sulfate overnight. After centrifugation at 10,000*g* for 20 min, the supernatant was pooled as a type V collagen fraction. To the precipitate, 0.5 M acetic acid containing 11.0% (w/v) ammonium sulfate was added and the suspension was stirred overnight. After centrifugation at 10,000*g* for 20 min, the supernatant was combined with the type V collagen fraction, while the resultant precipitate was collected as a type I collagen fraction. Types I and V collagens were purified from these fractions by phosphocellulose column chromatography according to the method reported by Yata et al. (2001) and used as the antigens in the subsequent preparation of antisera against these collagen types.

2.4. SDS–PAGE and peptide mapping

SDS–PAGE was performed by the method of Laemmli (1970) using 7.5% polyacrylamide gels. The samples (2–5 μg) were applied to sample wells and electrophoresed, along with type I collagen from carp *Cyprinus carpio* as a standard protein, whose alpha components have an approximate molecular weight of 100 kDa (Noda, Nagai, & Fujimoto, 1975). Gels were stained for protein with Coomassie Brilliant Blue (CBB) R-250, essentially as described by Fairbanks, Steck, and Wallach (1971). The gel was initially stained by soaking in 10% acetic acid containing 0.05% CBB R-250 and 25% 2-propanol for 1 h at room temperature. The first staining solution was then exchanged for 10% acetic acid containing 0.004% CBB R-250 and 10% 2-propanol. After 2 h, the gel was soaked in 10% acetic acid containing 0.002% CBB R-250 for 2 h. Then the background of the gel was extensively destained with 10% acetic acid. The collagen and related peptides were stained metachromatically and non-collagenous proteins were stained orthochromatically (Duhamel, 1983; Micko & Schlaepfer, 1978).

Peptide mapping with glutamyl endopeptidase from *Staphylococcus aureus*, strain V-8 (EC. 3.4.21.19, Sigma) was performed essentially as described by Cleveland, Fischer, Kirshner, and Laemmli (1977). The sample (5 μg), dissolved in 125 mM Tris–HCl, pH 6.8, containing 0.1% SDS, 1 mM EDTA, 0.02% bromophenol blue and 50% glycerol, was applied to the sample well and digested at an enzyme / substrate ratio of 1:10 (w/w) in the stacking gel. The digest was separated on 12.5% polyacrylamide gel and stained as above.

2.5. Preparation of antisera

Purified collagen was dissolved in 0.1 M acetic acid at a concentration of 1 mg/ml and the solution was emulsified in an equal volume of Freund's complete adjuvant. The emulsion (1 ml) was injected subcutaneously into the back of a rabbit. After 7 and 21 days the rabbit was further immunized with 1 ml of the same antigen. The rabbit was boosted subcutaneously in the same way as