

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

Shoshi Mizuta^{*}, Satoshi Fujisawa, Maki Nishimoto, Reiji Yoshinaka

Department of Marine Bioscience, Faculty of Biotechnology, Fukui Prefectural University, Obama, Fukui 917-0003, Japan

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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.

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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 col-

lagen 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

^{*} Corresponding author. Tel.: +81-770-52-6300; fax: +81-770-52-6003.

E-mail address: mizuta@fpu.ac.jp (S. Mizuta).

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 10,000g 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

10,000g 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 10,000g 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

above 7 days prior to the collection of blood. Blood was collected from the ear vein and left overnight in a cold room at 5 °C. After centrifugation, the resultant supernatant was used as an antiserum preparation.

2.6. Immunochemical analysis

After separation by SDS-PAGE, collagen chains were transferred to nitrocellulose membranes according to the method of Towbin, Staehelin, and Gordon (1979) and the membranes were immunostained by the avidin–biotin complex method, essentially as previously described (Mizuta et al., 1991). The nitrocellulose membranes were blocked with 2% casein in phosphate buffered saline (PBS; 10 mM sodium phosphate, pH 7.4, containing 150 mM NaCl) and then reacted with the primary antiserum against type I or V collagen from tiger puffer skin in a dilution of 1:100. The biotinylated goat anti-rabbit IgG antibody (Vector Lab., Burlingame, California, USA) was diluted 1:100, in PBS and used as the secondary antibody. The membranes were then reacted with the avidin–biotin complex, which had been prepared by mixing avidin and biotinylated horseradish peroxidase. The binding of antibodies was visualized with 0.06% 4-chloro-1-naphthol (Nacalai Tesque, Kyoto, Japan) or 0.04% diaminobenzidine tetrahydrochloride (Bio-Rad, USA) containing 0.006% H₂O₂.

3. Results and discussion

Fig. 1(a) shows the results of SDS-PAGE analysis of the RS-AL from several parts of tiger puffer. The patterns were quite similar among parts and similar to that of pepsin-digested type I collagen from carp white muscle electrophoresed as a standard protein, showing typical patterns of type I collagen with the bands of $\alpha 1(I)$, $\alpha 2(I)$ and β chains. As shown in Fig. 1(b), peptide maps of glutamyl endopeptidase digests were also sim-

ilar to each other. The existence of type I collagen in the skin and white muscle of tiger puffer was previously reported (Sato et al., 1997; Yata et al., 2001). These observation, and the present results, indicate that the major collagens in these parts can be identified as type I collagen.

In the SDS-PAGE analysis of the RS-AL, little information on the distribution of the minor collagen, type V, among the parts could be obtained, so we tried immunochemical detection of this minor collagen in the RS-AL. Pepsin-solubilized collagen was first prepared from the skin, which is relatively rich in collagen and fractionated into types I and V collagen fractions according to the method previously described (Yata et al., 2001). Fig. 2 shows the SDS-PAGE patterns of the fractions obtained. The SDS-PAGE pattern of the PSC was similar to that of the RS-AL with the bands of $\alpha 1(I)$ and $\alpha 2(I)$ chains. The insoluble fraction (type I collagen fraction) at 11% ammonium sulfate showed a similar SDS-PAGE pattern to that of the PSC, while the soluble fraction (type V collagen fraction) showed a pattern typical of type V collagen with the bands of $\alpha 1(V)$, $\alpha 2(V)$ and $\alpha 3(V)$ chains. These results indicate that type V collagen can be effectively concentrated in the soluble fraction by this method, as reported in the previous paper (Yata et al., 2001). Types I and V collagens were chromatographically purified from these fractions and the antisera were prepared against them. The reactivities of the antisera were then examined by immunoblot analysis (Fig. 3). The anti-type I collagen and anti-type V collagen sera showed very specific reactivity to the constituent subunits of types I and V collagens, respectively, under the conditions adopted. Fig. 4 shows the results of immunoblot analysis for the parts of tiger puffer, except for skin, using these antisera. Reactivity to these antisera was observed for the RS-AL from all the parts examined, the immunoblot patterns being quite similar between parts. The anti-type I collagen was mainly reactive for the $\alpha 1(I)$ chain, while the anti-type V collagen was for the component corresponding to $\alpha 1(V)$.

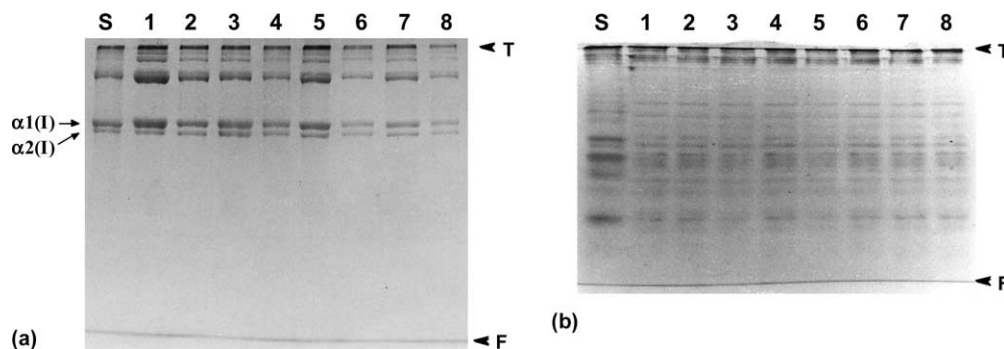


Fig. 1. SDS-PAGE analysis (a) and peptide maps of glutamyl endopeptidase digests (b) of the RS-AL from several parts of the tiger puffer. Lanes 1–8, RS-AL from skin, muscle, alimentary tract, liver, swim bladder, bone, fin and gill, respectively. Lane S, pepsin-digested type I collagen from carp white muscle. Letters T and F show the gel top and buffer front, respectively.

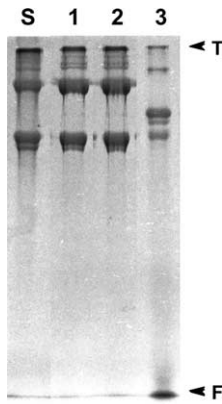


Fig. 2. SDS-PAGE analysis of pepsin-solubilized collagen (1) and the types I (2) and V (3) collagen fractions from the skin of tiger puffer, along with pepsin-digested type I collagen from carp white muscle (S). Letters T and F, as in Fig. 1.

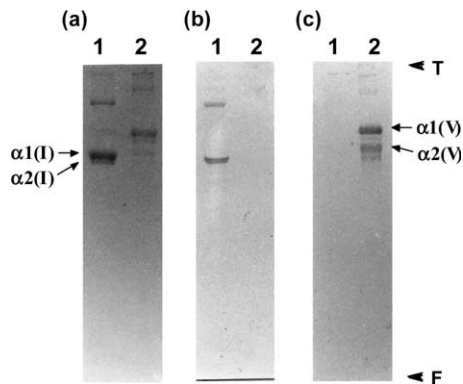


Fig. 3. SDS-PAGE (a) and immunoblot (b and c) analysis of types I (1) and V (2) collagens purified from pepsin-solubilized collagen preparation of the tiger puffer skin. The samples were separated on 7.5% polyacrylamide gels, transferred to nitrocellulose membrane and immunostained with the anti-type I (b) and anti-type V (c) collagen sera. Letters T and F, as in Fig. 1.

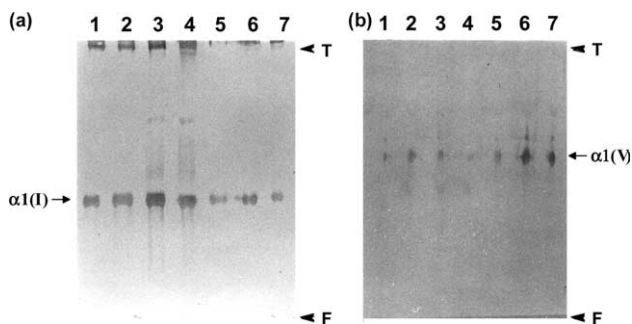


Fig. 4. Immunoblot analysis of the RS-AL from several parts of the tiger puffer, using the anti-type I (a) and anti-type V (b) collagen sera. The samples were separated on 5% polyacrylamide gels, transferred to nitrocellulose membrane and immunostained with the antisera. Lanes 1–7, RS-AL from muscle, alimentary tract, liver, swim bladder, bone, fin and gill, respectively. Letters T and F, as in Fig. 1.

These results suggest that types I and V collagens may be widely distributed in the parts of tiger puffer examined in the present study.

Sato et al. (1997) reported the presence of types I and V collagens in tiger puffer muscle, and contents in the muscle were determined to be 948 and 6.9 mg/100 g muscle, respectively. Yata et al. (2001) purified types I and V collagens from the skins of Japanese common mackerel, yellow sea bream and tiger puffer. However, the distribution of minor collagen types, such as type V, has never been reported in the parts except for muscle and skin of tiger puffer. In the present study, we detected the presence of type V collagen, as well as type I collagen, in several parts of tiger puffer by biochemical and immunochemical techniques. As for fish muscle, it was reported that enzymatic degradation of type V collagen in pericellular connective tissue is largely responsible for the *post-mortem* tenderization of muscle during chilled storage (Sato et al., 1997). In the case of tiger puffer muscle, Sato et al. (1997) also reported that little biochemical change occurred in type V collagen, which corresponded well to their observation that significant change in breaking strength of the muscle was hard to detect during chilled storage. Fish skin is also used as a foodstuff in Japan together with muscular tissue, to give a unique texture. From the present findings and these observations, it would be very interesting to investigate the function of type V collagen in the development of the texture of skin. For this purpose, it is necessary to obtain reliable information on the contents of types I and V collagens in the various parts of the fish.

The present results not only suggests the importance of type V collagen in the textural development of the edible parts of fish but also shows the potential of these parts of fish as resources of type V collagen for utilization. Further studies are now in progress to elucidate the distribution, content and biochemical properties of the collagen types in parts of several fish species, including tiger puffer.

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