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Existence of two molecular species of collagen in the muscle layer of the ascidian (*Halocynthia roretzi*)

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Abstract

The molecular species of collagen in the muscle layer of the ascidian *Halocynthia roretzi* was examined by biochemical techniques. Two types of collagen which showed distinct patterns from each other on SDS-PAGE were isolated from the pepsin-solubilized collagen by differential salt precipitation and phosphocellulose column chromatography. They were demonstrated to be genetically distinct from each other by peptide mapping and by amino acid analysis. These results indicate that at least two molecular species of collagen are present in the muscle layer of the ascidian.

Keywords: Collagen; Sea squirt; Ascidian; Muscle; Connective tissue

1. Introduction

Collagen is one of the fundamental components comprising muscle connective tissue of multicellular animals. It has recently been reported that muscle collagen is very important in the development of the texture of raw or cooked meat from several aquatic animals (Mizuta, Yamada, Miyagi, & Yoshinaka, 1999; Mizuta, Yoshinaka, Sato, & Sakaguchi, 1994a, 1997; Olaechea, Ushio, Watabe, Takeda, & Hatae, 1993; Sato et al., 1997). Minor collagens, especially, have received increased attention as important constituents affecting post mortem textural change of fish meat during chilled storage. The phenomenon of muscle tenderization has been suggested to arise from disintegration of pericellular collagen fibres due to proteolytic breakdown of telopeptides of Type V collagen (Ando, Yoshimoto, Inabu, Nakagawa, & Makinodan, 1995; Sato et al., 1997).

Ascidians are accepted as being one of the commercially important groups of aquatic animals that are used as food resources in Korea, Japan, and other Asian countries. The ascidian *Halocynthia roretzi* is the main species used as food and is cultured extensively in northeastern Japan and southern Korea. As for collagen in ascidian animals, Pikkarainen, Rantanen, Vastamäki, Lampiaho, Kari, and Kulonen (1968) reported the content and solubility of collagen in the body wall of the ascidian *Ciona intestinalis*. Kimura, Kobayashi, and Kubota (1972) prepared pepsin-solubilized collagen from the muscle layer of the ascidian, *Halocynthia roretzi*, and reported its physicochemical and biochemical properties. However, little information is available on the molecular species of collagen in ascidian tissues. In the present study, we tried to fractionate pepsin-solubilized collagen from the muscle layer of the ascidian and partially characterize each molecular species of collagen, in order to obtain fundamental information for elucidating the function of collagen in the development of the meat texture of the ascidian.

2. Materials and methods

2.1. Preparation and fractionation of collagens

Cultured ascidian, *Halocynthia roretzi* (average body weight, 269 g), were obtained alive from a nursery at Miyagi Prefecture, Japan, in April 1997. Muscle layer tissues (about 500 g) were dissected out from the bodies, homogenized in 5 volumes (v/w) of 0.1 M NaOH, and extracted in the same solution for 24 h with gentle stirring

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at 5 °C. The extraction was done to remove non-collagenous proteins and to prevent the effect of endogenous proteases on collagen, as described previously (Yoshinaka, Mizuta, Itoh, & Sato, 1990). The residue after alkali extraction (RS-AL) was thoroughly washed with distilled water, and 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 (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 (PS) collagen preparation.

The PS collagen was salted out, collected by centrifugation at 10,000 ***g*** for 20 min, suspended in distilled water, and neutralized by dialysis against 20 mM Na_2HPO_4 to inactivate pepsin. After centrifugation at 10,000 ***g*** for 20 min, the precipitated PS collagen was extracted with 0.5 M acetic acid containing 10% (w/v) ammonium sulfate. The soluble and precipitable fractions were separated from each other by centrifugation at 10,000 ***g*** for 20 min, and referred to as S-10 and P-10 fractions, respectively.

2.2. Phosphocellulose column chromatography

The S-10 fraction was further fractionated on a column of phosphocellulose (Whatman P11) in a cold room at 5 °C. The collagen in the S-10 fraction was salted out by adding ammonium sulphate to a final concentration of 20% (w/v) and dialyzed against a starting buffer (50 mM sodium acetate, pH 4.8, containing 2.0 M urea and 0.1 M NaCl). After complete dialysis, the sample was applied to the column (2.5 × 5 cm) which had been equilibrated with the same starting buffer. Adsorbed proteins were eluted with a linear gradient of 0.1–1.0 M NaCl at a flow rate of 60 ml/h. The effluent was monitored at 230 nm by a UV–Vis detector (UV-9900, Tokyo Rikakikai, Tokyo, Japan). Appropriate fractions were pooled, dialyzed against distilled water, and lyophilized.

2.3. Analytical methods

SDS-PAGE was performed by the method of Laemmli (1970) using 4.5 or 5% polyacrylamide gels. The samples (about 5 μg) were applied to sample wells and electrophoresed, along with Type I collagen from carp *Cyprinus carpio* muscle as a standard protein, of which alpha components have an approximate molecular weight of 100 kDa (Noda, Nagai, & Fujimoto, 1975) and mixture of standard proteins (SDS-6H, Sigma Chemical Corporation, St. Louis, Mo., USA). 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). Approximate relative staining intensity of alpha components was estimated using an image analysis software (Mac Scope, Mitani Corporation, Fukui, Japan).

Peptide mapping with endoproteinase Glu-C from *Staphylococcus aureus* strain V-8 (V-8 protease, EC.3.4.21.19, Sigma) or lysyl endopeptidase from *Achromobactor lyticus* (EC.3.4.21.50, Wako, Osaka, Japan) was performed essentially as described by Cleaveland, Fischer, Kirshner, and Laemmli (1977). The sample (about 5 μg) dissolved in 125 mM Tris–HCl, pH6.8, 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 10 or 12.5% polyacrylamide gel, and stained as earlier.

The amino acid composition was analyzed by a PICO TAG amino acid analysis system (Waters, USA). Samples were hydrolyzed in vacuo in 6 M HCl 150 °C for 1 h.

3. Results and discussion

Although collagen from the muscle layer of the ascidian was quite insoluble in 0.5 M acetic acid, more than 80% of the total collagen was solubilized by limited pepsin digestion. The resultant PS collagen was fractionated into two fractions, S-10 and P-10, by ammonium sulfate precipitation. Approximately 10% of the total PS collagen was soluble in 10% ammonium sulfate (S-10 fraction), while the rest was insoluble (P-10 fraction). SDS-PAGE patterns of these fractions are shown in Fig. 1, along with Type I collagen from carp white muscle. The PS collagen showed a main alpha band, chain **a**, with mobility on SDS-PAGE, which was comparable with that of α1(I) of carp Type I collagen, and a few faint bands. The P-10 fraction showed essentially a similar pattern to that of the PS collagen. In the pattern of the S-10 fraction, however, other alpha chain-sized components, designated as **b** and **c**, were observed together with the chain **a**, beta chain-sized components, and non-collagenous component near the buffer front. The S-10 fraction eluted as three peaks on phosphocellulose column chromatography (Fig. 2). The first peak (unadsorbed fraction) contained little collagenous component when examined by SDS-PAGE (data not shown). Both of the adsorbed fractions (fractions A

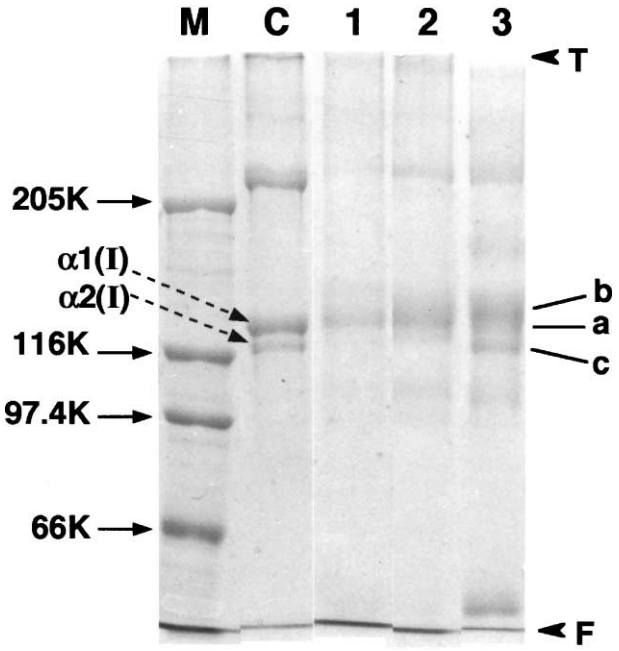

Fig. 1. SDS-PAGE analysis (5% gel) of the pepsin-solubilized collagen (lane 1), and P-10 fraction (lane 2), and S-10 fraction (lane 3), along with molecular weight standard (lane M) and carp Type I collagen (lane C). The molecular weight standard shows four bands of myosin from rabbit muscle (205 kDa), β-galactosidase from *Escherichia coli* (116 kDa), phosphorylase b from rabbit muscle (97.4 kDa), and bovine serum albumin (66 kDa). Arrowheads T and F show the gel top and buffer front, respectively.

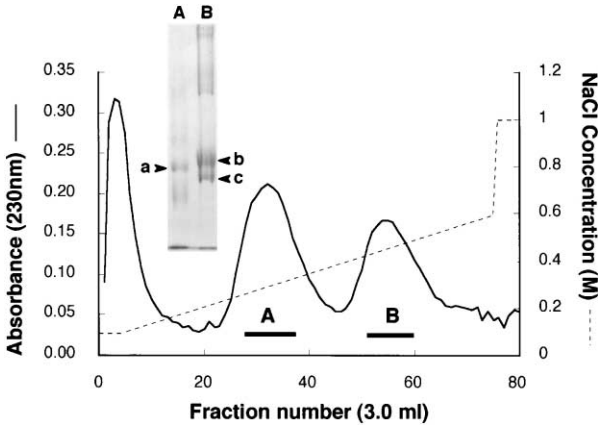

Fig. 2. Phosphocellulose column chromatography of the S-10 fraction. The column (2.5 × 5 cm) was equilibrated with 50 mM sodium acetate, pH 4.8, containing 2.0 M urea and 0.1 M NaCl. Adsorbed proteins were eluted with a linear gradient of 0.1–1.0 M NaCl over a total volume of 210 ml at a flow rate of 60 ml/h. The inset shows the SDS-PAGE patterns (4.5% gel) of the fractions A (lane A) and B (lane B).

and B) showed almost symmetrical peaks with no shoulder, and contained collagenous components presenting metachromasy on SDS-PAGE. The fraction A showed the bands of chain **a** and some lower molecular weight components, exhibiting a similar pattern to those of the PS collagen and P-10 fraction. On the other hand, the fraction B showed dense bands of the chains **b** and **c**, and beta chain-sized components. Relative staining intensity of the chain **b** to **c** was estimated to be about 2:1 by the image analysis system. The alpha components, **a**, **b** and **c**, which comprised collagen molecules in the fractions A and B, were electrophoretically recovered from the gel slices and subjected to peptide mapping with V-8 protease and lysyl endopeptidase (Fig. 3). As shown in Fig. 3–1, each alpha component was successfully recovered, although the staining intensity of the chain **c** was rather faint (Fig. 3–1, lane c). The chains **a**, **b**, and **c** showed different patterns from one another in peptide mapping with both V-8 protease (Fig. 3–2) and lysyl endopeptidase (Fig. 3–3). These combined results indicate that the fractions A and B in the phosphocellulose chromatography contain genetically distinct collagen types from each other. These collagen types were temporarily termed Type AS-I and AS-II collagens (AS: Ascidian), respectively. The P-10 fraction had quite low solubility in various starting conditions of ion-exchange chromatography, so we could not perform ion-exchange chromatography as a purifying method for this major fraction in the present study.

Table 1 shows the amino acid composition of Type AS-I and AS-II collagens. Notable differences were found in the content of alanine, isoleucine, and hydroxylysine between Type AS-I and AS-II collagens, supporting the occurrence of genetical difference between them. It is of especial interest that the alanine contents of Type AS-I and AS-II collagens are considerably lower than those of muscle collagens from other aquatic animals reported, such as fish (Kimura, Zhu, Matsui, Shijoh, & Takamizawa, 1988; Sato, Yoshinaka, Itoh, & Sato, 1989), prawns (Minamisako & Kimura, 1989; Yoshinaka et al., 1990), cephalopod mollusks (Mizuta, Yoshinaka, Sato, & Sakaguchi, 1994b; Takema & Kimura, 1982), and gastropod mollusks (Kimura & Kubota, 1968; Kimura & Tanaka, 1983). Half-cystine was not detected in either Type AS-I or AS-II collagen. In our additional experiments, the reduction with 2-mercaptoethanol did not affect the SDS-PAGE patterns of Type AS-I and AS-II collagens (data not shown). These results suggest the lack of disulfide bonds in these collagen molecules.

In the present study, the isolation of two types of collagen, AS-I and AS-II, from the muscle layer of the ascidian was successfully achieved by differential salt precipitation and phosphocellulose column chromatography, while the isolation of collagen types in the major fraction, P-10, remained to be done with a problem of its poor solubility under native conditions. Type AS-I collagen showed the alpha chain **a** and some bands of lower molecular weight components on SDS-PAGE, implying a possibility that this type might undergo some structural change or degradation in the triple helical

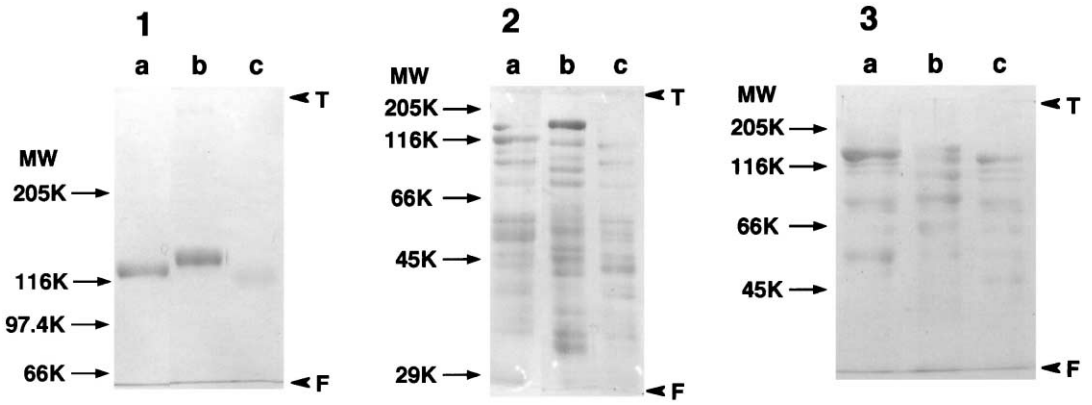

Fig. 3. SDS-PAGE analysis (1, 4.5% gel) and peptide maps of V-8 protease (2, 12.5% gel) and lysyl endopeptidase (3, 10% gel) digests of the chains **a** (**a**), **b** (**b**), and **c** (**c**) which were electrophoretically recovered from the gel slices. Arrows (MW 205 kDa, 116 kDa, 97.4 kDa, 66 kDa, 45 kDa, and 29 kDa) show the molecular weights and positions of the standard proteins. Arrowheads T and F, as in Fig. 1.

Table 1
Amino acid composition of Type AS-I and AS-II collagens from the muscle layer of ascidian (residues/1000 residues)

	Type AS-I	Type AS-II
Hydroxyproline	82.1	74.1
Aspartic acid	48.9	52.7
Threonine	38.0	45.4
Serine	64.1	65.0
Glutamic acid	72.0	59.3
Proline	78.8	78.7
Glycine	356.5	362.7
Alanine	27.0	37.6
Valine	22.8	28.3
Methionine	12.6	9.4
Isoleucine	33.6	19.1
Leucine	36.9	36.0
Tyrosine	6.6	5.3
Phenylalanine	14.6	10.8
Hydroxylysine	27.5	37.1
Lysine	15.1	19.1
Histidine	6.9	10.0
Arginine	56.1	49.4

region by pepsin digestion. On the other hand, Type AS-II collagen showed a pattern of the typical collagen molecule, like vertebrate Type I, exhibiting two alpha bands, of which the relative staining intensity was about 2:1, and beta and gamma components. This collagen type was effectively concentrated in the minor fraction, S-10, by ammonium suphate precipitation, showing a similar solubility to those of fish Type V (Mizuta, Hwang, & Yoshinaka, 2002; Sato et al., 1989; Yata, Yoshida, Fujisawa, Mizuta, & Yoshinaka, 2001) or prawn Type AR-Ib and AR-Ic (Mizuta, Yoshinaka, Sato, Itoh, & Sakaguchi, 1992; Mizuta, Yoshinaka, Sato, Suzuki, Itoh, & Sakaguchi, 1991). In addition, the amino acid composition of this type had a very low content of alanine (37.6 residues/1000 residues) and very high content of hydroxylysine (37.1 residues/1000 residues), almost comparable with those of the α2(AR-I) component of Type AR-Ib or AR-Ic collagen in kuruma prawn (Mizuta et al., 1991). These facts suggest that Type AS-II collagen may correspond to vertebrate Type V collagen or crustacean Type AR-Ib or AR-Ic collagens. Further studies are now in progress to elucidate the nature of molecular species in the major fraction, P-10, and the subunit composition of Type AS-I and AS-II collagens.

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