Immunochemical and Immunohistochemical Identification of a Minor Collagen in Raw Muscles of Decapod Mollusks

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ABSTRACT: Approximately 10% of total muscle collagen in 6 decapod molluskan species was recovered as minor collagenous fractions by limited pepsin digestion and differential salt precipitation. The main alpha components (α1) in these fractions showed similar peptide maps of V-8 protease and lysyl endopeptidase digests to each other and had reactivity to the antiserum against the α1 component of the minor collagen (named Type SQ-II) from *Todarodes pacificus* muscle in immunoblot analysis. These results suggested that the minor molecular species of collagen corresponding to Type SQ-II of *Todarodes pacificus* was widely distributed in the mantle muscle of decapod mollusks. In addition, immunohistochemical experiments revealed that Type SQ-II collagen distributed mainly in intramuscular thin connective tissue (endomysium), suggesting the functional importance of this molecule to the development of meat texture of decapod mollusks.

Key Words: collagen, muscle, mantle, squid, cuttlefish, connective tissue

Introduction

MUSCLE CONNECTIVE TISSUES OF multicellular animals contain collagen as a fundamental protein, which plays mechanically and physiologically important roles. Quantitatively minor collagens have recently received increased attention as important constituents affecting postmortem physical properties of meat, such as texture or firmness, of aquatic animals, especially of fish. The phenomenon of muscle tenderization during chilled storage has been suggested to arise from disintegration of pericellular collagen fibers due to proteolytic degradation of telopeptides of Type V collagen (Ando and others 1992, 1993, 1995; Sato and others 1997). It was reported that an increased solubility of Type V collagen during chilled storage accounted for the enzymatic degradation of Type V collagen of fish muscle (Sato and others 1991, 1994).

In spite of such an importance, information on quantitatively minor collagens of decapod mollusk is quite limited. We have recently reported the presence of a minor collagen, named Type SQ-II, which composed about 10% of the total collagen in the mantle and skin of squid *Todarodes pacificus* as well as a major collagen, Type SQ-I (Mizuta and others 1994c). Both types of collagen were heterotrimers with subunit compositions corresponding to $[\alpha1(SQ\text{-}I)]_2\alpha2(SQ\text{-}I)$ and $[\alpha1(SQ\text{-}II)]_2\alpha2(SQ\text{-}II)$, respectively (Mizuta and others 1994a). The purpose of the present study was to clarify the distribution of the minor collagen among several decapod species by biochemical and immunochemical techniques and to elucidate the histological distribution of this molecule in mantle tissue.

Results and Discussion

WE PREVIOUSLY DEMONSTRATED THE presence of 2 genetically distinct types of collagen in the mantle muscle, mantle skin, and arm muscle of the squid *T. pacificus*, which were named Type SQ-I and SQ-II collagens (Mizuta and others 1994c). Because of insolubility of the squid collagen, they could be isolated only from the pepsin-solubilized collagen preparation. In the present study, we first prepared RS-ALs and pepsin-solubilized collagens from the mantle muscles of the 6 decapod species and tried to detect components related to α1(SQ-II) component in them by SDS-PAGE and immunoblot analysis. Figures 1A and 1B show the SDS-PAGE patterns and the immunoblot analysis performed using the anti-α1(SQ-II) component serum that had been prepared and characterized (Mizuta and others 1995). The RS-AL from *T. pacificus* showed 4 alpha chain-sized components, designated as chains **a**, **b**, **c**, and **d**, and several higher molecular weight components. The RS-ALs from the other species also showed chains corresponding to these chains, although the band of **b** chain from some species was fairly weak (Fig. 1A). In immunoblot analysis, the chains that corresponded to the chain **a** of *T. pacificus* were reactive for the anti-α1(SQ-II) component serum (Fig. 1B). As shown in Fig. 1C, the SDS-PAGE patterns of pepsin-solubilized collagens from the 5 species were similar to that of *T. pacificus*, showing the bands corresponding to α1(SQ-II) and α1(SQ-I), and to α2 component, which had been shown to contain both α2(SQ-I) and α2(SQ-II) components (Mizuta and others 1994a, 1996, 1997). In any species, the mobility of the α1(SQ-II)-corresponding component was larger than that of the chain **a** and almost identical to that of the chain **b**, probably due to degradation of relatively large telopeptides by pepsin digestion. Immunoblot analysis of the pepsin-solubilized collagens showed a clear reactivity of the anti-α1(SQ-II) component serum to the α1(SQ-II)-corresponding components of all the species examined (Fig. 1D).

In the fractionation of the pepsin-solubilized collagen with 0.5M acetic acid containing 0.45M NaCl, a small amount of collagen was obtained in the soluble fraction (S-0.45 fraction) for each species and showed a similar SDS-PAGE pattern to each other (Fig. 1E). When the fractions obtained were subjected to immunoblotting using anti-α1(SQ-II) component serum, only the α1(SQ-II)-corresponding components were evidently reactive (Fig. 1F). SDS-PAGE patterns of the S-0.45 fractions were not affected by the presence of 2-mercaptoethanol (data not shown), suggesting the lack of disulfide bond in the collagen molecule of these fractions. On the other hand, the insoluble fractions (P-0.45 fractions) exhibited similar SDS-PAGE patterns to each other and to those of the pepsin-solubilized collagens shown in Fig. 1C (data not shown). The α1(SQ-II)-corresponding components were recovered from gel slices, and peptide maps of their V-

8 protease and lysyl endopeptidase digests were compared among species (Fig. 2). Peptide maps of the α1(SQ-II)-corresponding components were quite similar to each other, indicating the similarity of their primary structure.

Shadwick (1985) suggested the presence of at least 2 molecular species of collagen in the skin, tunic, and mantle muscle of the squid *Loligo vulgaris* and cuttlefish *Sepia officinalis*. However, identification of each molecular species remained to be established in decapod mollusks, except for our work on *T. pacificus* (Mizuta and others 1994c). In the present study, collagenous material recovered in S-0.45 fractions (Type SQ-II collagen fractions) of the 6 decapod species showed similar SDS-PAGE patterns to each other. Moreover, the α1(SQ-II)-corresponding components from the 6 species were specifically reactive for the anti-α1(SQ-II) component serum, and exhibited similar peptide maps of V-8 protease digests to each other. These combined observations suggested that the minor molecular species of collagen corresponding to Type SQ-II collagen might be widely distributed in the mantle muscle of decapod mollusks. We, therefore, propose that this type of collagen should be unified into or represented as Type SQ-II collagen. As for quantitatively major molecular species, we have characterized a major collagen in the mantle muscle of *T. pacificus*, named Type SQ-I collagen (Mizuta and others 1994c). We have not succeeded in preparation of antiserum against Type SQ-I collagen with high specificity and titer.

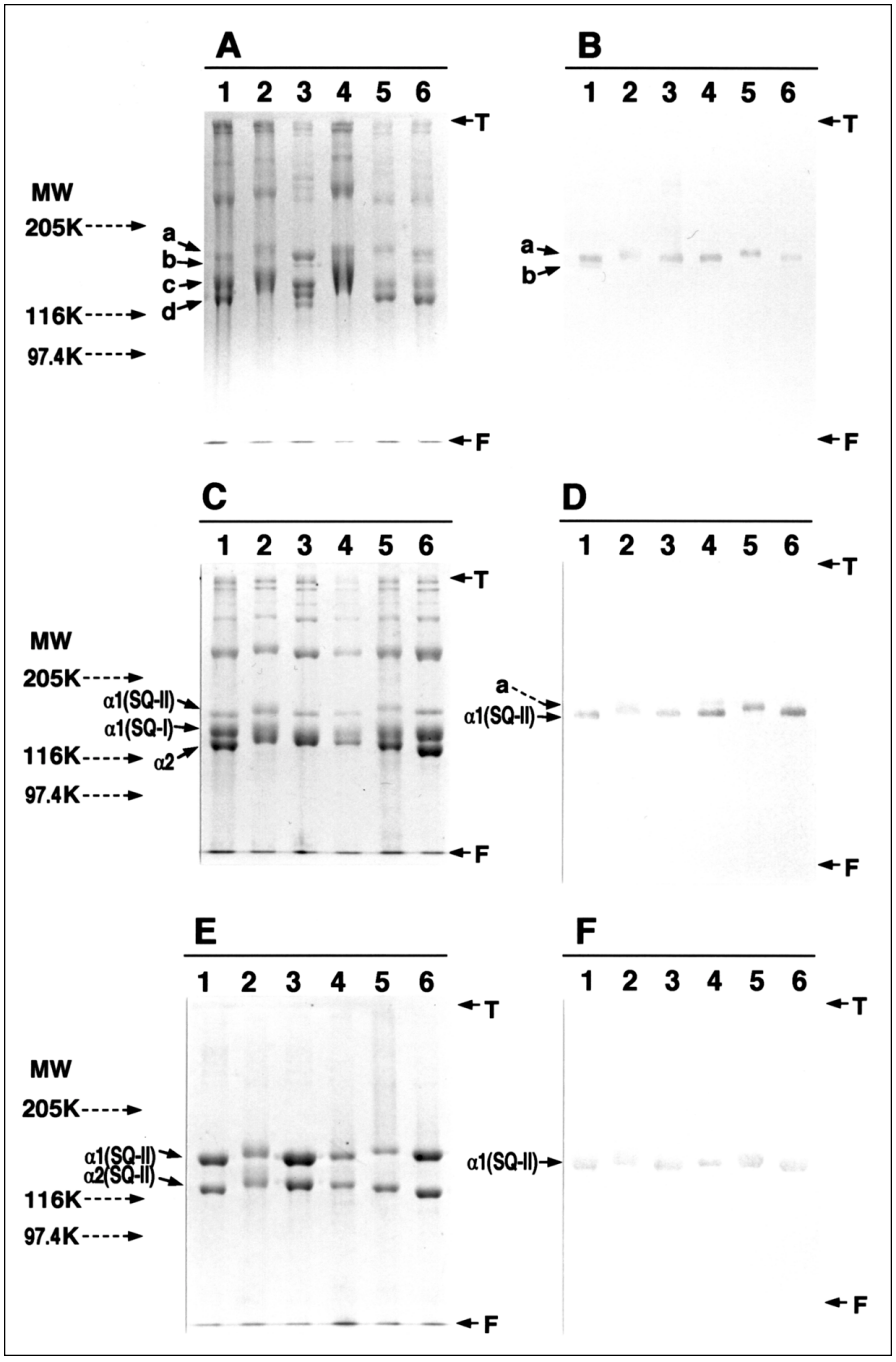

Fig. 1—SDS-PAGE patterns (5% gel) (A, C, and E) and anti-α1(SQ-II) immunoblot analysis (B, D, and F) of the residual fractions after alkali extraction (A and B), pepsin-solubilized collagens (C and D), and S-0.45 fractions (E and F) of 6 decapod species. Species represented in lanes 1 to 6 are *Todarodes pacificus*, *Loligo edulis*, *Sepioteuthis lessoniana*, *Thysanoteuthis rhombus*, *Sepia esculenta*, and *Sepia longipes*, respectively. Arrows a, b, c, and d show the positions of the chain a, b, c, and d, respectively. Arrows MW designate the mobility of standard proteins, myosin heavy chain (205K, from rabbit muscle), β-galactosidase (116K, from *Escherichia coli*), and phosphorylase b (97.4K, from rabbit muscle). Letters T and F indicate the gel top and dye front, respectively.

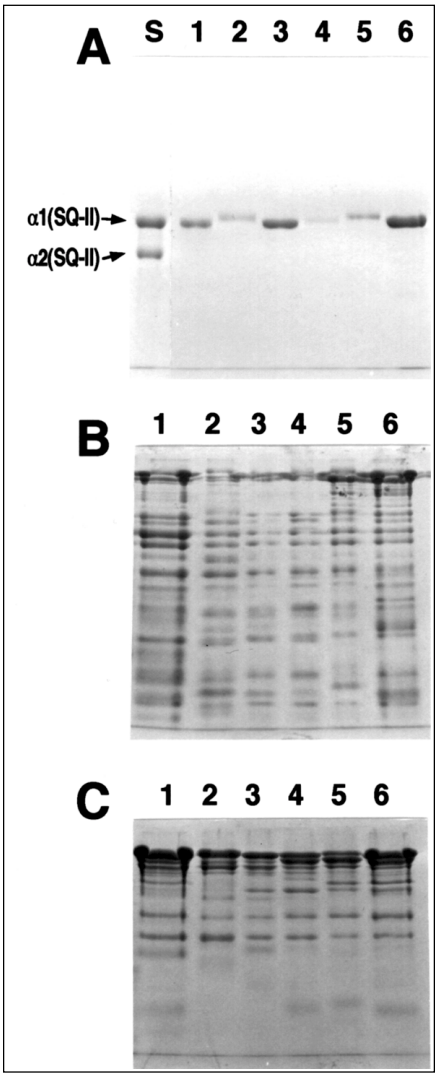

Fig. 2—SDS-PAGE (5% gel) of the α1(SQ-II)-corresponding components from 6 decapod species (A) and their peptide maps of V-8 protease (B, 12.5% gel) and of lysyl endopeptidase (C, 10% gel) digests. Lane S, S-0.45 fraction from *Todarodes pacificus*. Species represented in lanes 1 to 6, as in Fig. 1.

Therefore, immunochemical detection of the major collagen corresponding to Type SQ-I collagen remained to be done. In the present study, however, major collagenous fractions from the 6 species showed quite a similar behavior on differential NaCl fractionation, precipitating in 0.45M NaCl solution at acidic pH. Moreover, peptide maps of lysyl endopeptidase digests of the α1(SQ-I)-corresponding components from the 6 species showed a high similarity to each other in a preliminary experiment (data not shown). From these observations, the major molecular species of collagen that corresponds to Type SQ-I may also be widely distributed in decapod mollusks.

Because of the high insolubility of squid and cuttlefish collagens, Type SQ-II collagen could be isolated only from pepsin-solubilized collagen preparation as in the previous (Mizuta and others 1994b) and present data. Therefore, effects of protease digestion on Type SQ-II collagen should be taken into consideration in association with changes of biochemical properties of Type SQ-II collagen. Moreover, it seems to be of special interest to clarify this problem from the viewpoint of effects of muscle endogenous proteases on collagen in association with postmortem changes in physical properties of meat. We previously demonstrated that intact (not pepsinized) form of the α1(SQ-II) component from *T. pacificus* (the chain **a**) had a larger molecular weight probably due to having relatively large telopeptides (Mizuta and others 1996). The present data showed the wide presence of intact forms of the α1(SQ-II) components with relatively large telopeptides in decapod animals. The intact molecule of Type SQ-II collagen from the mantle muscle of *T. pacificus* was previously found to be preferentially solubilized by 4M guanidine hydrochloride, and an intact form of the α2(SQ-II) component was suggested to have higher molecular weight than α2(SQ-II) component as in the case of the α1(SQ-II) component (Mizuta and others 1997). However, solubility and biochemical properties of the intact form of Type SQ-II collagen of decapod species remain to be investigated.

We next tried to clarify the histological distribution of the minor collagen Type SQ-II in the mantle of squid, focusing on that of *T. pacificus*, by immunohistochemical staining. Tanaka (1958) described the histological structure of mantle of decapod mollusks. According to this report, mantle muscle of squid consists of 2 kinds of layers, named muscle layers A and B. Muscle layer A runs circularly around the longitudinal axis and occupies a large part of the total muscular tissue. On the other hand, muscle layer B runs perpendicularly to the muscle layer A. Figures 3A, 3B, 3C, and 3D show the results of Van Gieson stain of the prepared sections from the mantle of *T. pacificus*. The outer side of the skin tissue can be divided into 3 layers, which are epidermal connective tissue (EC), chromatophore layer (CL), and dermal connective tissue (DC), while a layer of dermal connective tissue was observed at the inner skin tissue. Intramuscular connective tissue consists of epimysium (EP) and endomysium (EN). Epimysium is a thick connective tissue that forms the outer sheath of the muscular tissue, while endomysium are thin collagen fibers scattering in the inner part of muscular tissue. Collagen fibers of endomysium are heterogeneous in their distribution density, diameter, and arrangement direction. It was often observed that relatively thick fibers were distributed densely near the outer epimysium (Fig. 3B), and that relatively thin fibers were scattered sparsely around the central part of the muscle (Fig. 3C). Collagen fibers that ran almost perpendicularly to muscle fibers were also observed with smaller density. The mantle tissue of the other 5 species showed quite a similar structure to that of *T. pacificus* (data not shown). In immunohistochemical staining with the anti-α1(SQ-II) component serum, intense positive reaction was detected in epidermal connective tissue (Fig. 3E), dermal connective tissue (Fig. 3E and 3H), and endomysium (Fig. 3F and 3G), while the reactivity to the epimysium was faint (Fig. 3E, 3F, and 3H). These results suggested that Type SQ-II collagen might be mainly distributed in the endomysium for

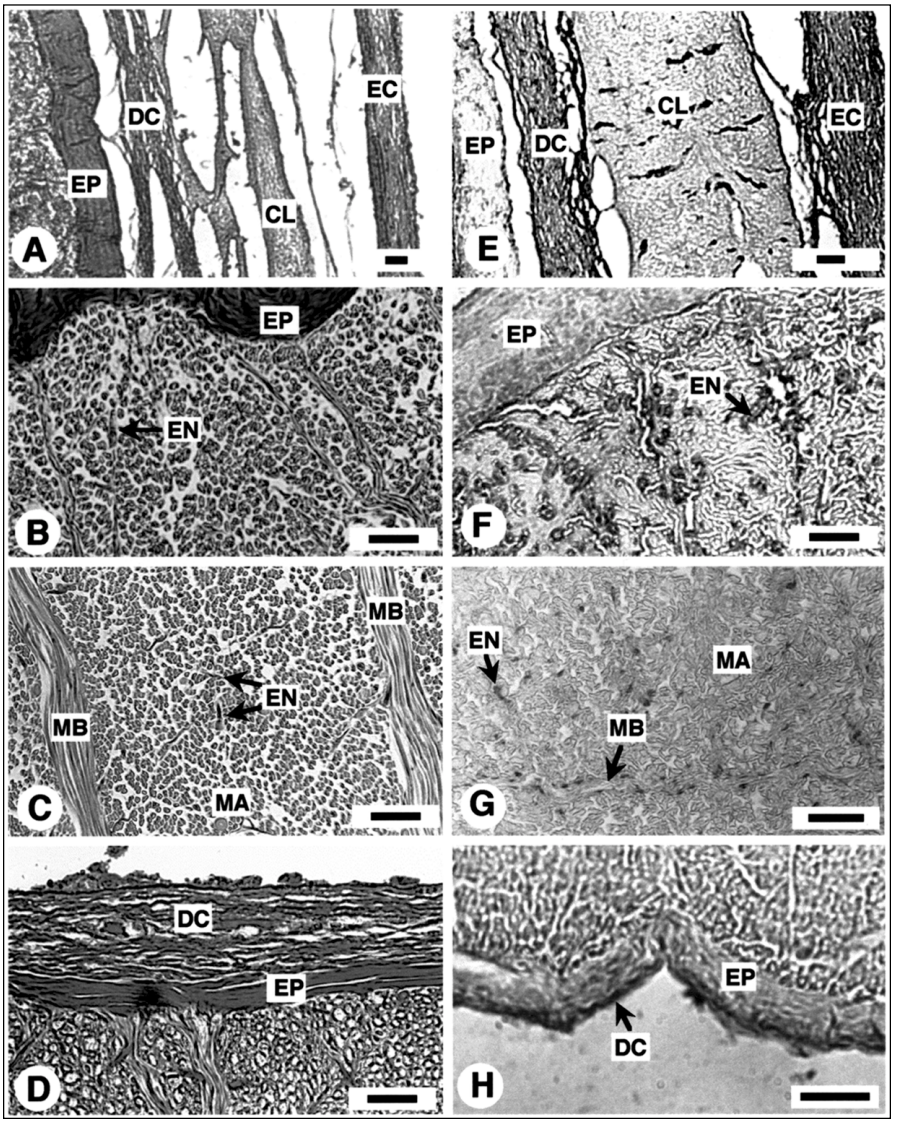

Fig. 3—Light micrographs of the sections, parallel to the longitudinal axis, of *Todarodes pacificus* mantle. A, B, C, and D, stained with Van Gieson stain; E, F, G, and H, immunostained with the anti-α1(SQ-II) component serum. A and E, outer side of skin tissue; B and F, muscular tissue near the epimysium of outer side; C and G, central part of muscular tissue; D and H, inner side of skin tissue. Letters EC, CL, DC, EP, EN, MA, and MB indicate the positions of epidermal connective tissue, chromatophore layer, dermal connective tissue, epimysium, endomysium, muscle layer A, and muscle layer B, respectively. Bars = 25μm.

muscular tissue, and in epidermal and dermal connective tissues for skin tissue.

It was reported that Type SQ-II collagen had not only a similar solubility in differential salt precipitation but a similar amino-acid composition to those of fish Type V collagen (Mizuta and others 1994c), showing a lower content of alanine and higher content of hydroxylysine. Type V collagen has been reported to be distributed mainly in endomysium of bovine muscle for mammalian animals (Bailey and others 1979). In fish species, existence of Type V collagen has been suggested for the pericellular thin connective tissue of sardine *Sardinops melanosticta* muscle (Sato and others 1997) by electron microscopic techniques. In addition, degradation of Type V collagen has been suggested to cause disintegration of thin collagen fibrils in pericellular thin connective tissue, resulting in postharvest muscle softening (Sato and others 1997).

Conclusion

These facts lead us to a consideration that Type SQ-II collagen corresponds functionally to vertebrate Type V collagen and may have a role in the development of squid meat texture.

Materials and Methods

Preparation of collagens

Several species of squid and cuttlefish, listed in Table 1, were obtained fresh within several hours after death from a local market. The muscle from each species (about 500 g) was dissected from the mantle, homogenized in 5 volumes (v/w) of 0.1 N NaOH, and stirred for 24 h at 5 °C. These procedures were done to remove noncollagenous proteins and to prevent the effect of endogenous proteases on collagen as described previously (Yoshinaka and others 1990). The residual fraction after the alkali extraction (RS-AL) was washed thoroughly with distilled water, lyophilized, and used as an RS-AL preparation.

Table 1—Data of samples

Species[a]	Average body weight (g)	Average mantle length (cm)	Month of sampling
Oegopsida			
Todarodes pacificus (10)	411.6	26.5	JAN
Thysanoteuthis rhombus (3)	1561.1	34.3	SEP
Myopsida			
Loligo edulis (10)	130.2	13.9	SEP
Sepioteuthis lessoniana (10)	111.7	12.4	SEP
Sepioidea			
Sepia esculenta (10)	334.3	15.9	APR
Sepia longipes (10)	324.4	17.7	SEP

[a]Numbers of individuals obtained were shown in the parenthesis

The RS-AL was digested with porcine pepsin (EC 3.4.23.1, crystallized and lyophilized, Sigma, St. Louis, Mo., U.S.A.) in 10 volumes (v/v) of 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 pepsin-solubilized collagen. The pepsin-solubilized collagen was salted out by adding NaCl to a final concentration of 2 M at 5 °C. The resultant precipitate was collected by centrifugation at 10,000 × g for 20 min and further extracted with 10 volumes (v/v) of 0.5M acetic acid containing 0.45M NaCl. The soluble matter, termed S-0.45 fraction, was salted out by adding NaCl to 2M, dialyzed against 0.02M Na_2HPO_4 and successively against distilled water, and lyophilized. In addition, the insoluble fraction (P-0.45 fraction) was washed with 0.05M Tris-HCl, pH 7.5, containing 2.4 M NaCl. The insoluble matter was dialyzed against 0.02M Na_2HPO_4 and successively against distilled water, and lyophilized.

SDS-PAGE and peptide mapping

SDS-PAGE was performed by the method of Laemmli (1970) using 5% polyacrylamide gels in the presence or absence of 1% 2-mercaptoethanol. Proteins were suspended (1mg/mL) in 0.05M Tris-HCl, pH 7.5, containing 0.1% SDS, 30% glycerol, and 0.02% bromophenol blue, and denatured at 80 °C for 10 min. The sample solution (5 μL) was applied to a sample well and electrophoresed. Gels were stained for protein with Coomassie Brilliant Blue (CBB) R-250 essentially as described by Fairbanks and others (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 noncollagenous proteins were stained orthochromatically (Duhamel 1983; Micko and Schlaepfer 1978).

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 *Achromobacter lyticus* M 497-1 (EC 3.4.21.50, Wako, Osaka, Japan) was performed essentially as described by Cleveland and others (1977). Proteins were dissolved (1mg/mL) in 125mM Tris-HCl, pH 6.8, containing 0.1% SDS, 1mM EDTA, 0.02% bromophenol blue, and 50% glycerol. The sample solution (5 μL) was applied to the sample well and digested with proteases at an enzyme/substrate ratio of 1:10 (w/w) in the stacking gel. The V-8 protease and lysyl endopeptidase digests were separated on 12.5% and 10% gels, respectively, and stained as above.

Immunochemical analysis

After separation by SDS-PAGE, collagen chains were transferred to nitrocellulose membranes according to the method of Towbin and others (1979), and the membranes were immunostained by the avidin-biotin complex method essentially as previously described (Mizuta and others 1991). The nitrocellulose membranes were blocked with 2% hen egg ovalbumin in phosphate buffered saline (PBS; 10 mM sodium phosphate buffer, pH 7.4, containing 150 mM NaCl) and then reacted with a primary antiserum against α1(SQ-II) component from *T. pacificus* previously prepared (Mizuta and others 1995) in dilution of 1:100 to about 1:1000. The biotinylated goat anti-rabbit IgG antibody (Vector Lab, Burlingame, Calif., U.S.A.) was diluted in 1:1000 to about 1:250 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, containing 0.006 % H_2O_2.

Histological observations

Histological observation by a light microscope was carried out by the same method as in the previous report (Mizuta and others 1994b). Small pieces of the muscular tissue were dissected,

fixed in Bouin's solution (Hoshino and others 1998) for 6 h, and embedded in paraffin (Parahisto, Nacalai Tesque, Japan). Four-micrometer sections were cut with a microtome. The prepared sections were stained with Van Gieson stain (Hoshino and others 1998) and observed with a light microscope (OPTIPHOTO-2, Nikon, Japan). Muscle fibers and collagen fiber were stained yellow and red, respectively. For immunohistochemistry, the sections prepared as above were first incubated in 3.5% H_2O_2 for 10 min to inhibit the endogenous peroxidase activity. After blocking with normal goat serum, sections were reacted with primary antiserum for 30 min at room temperature. The slides were washed with PBS for 10 min and then reacted with the biotinylated goat anti-rabbit IgG for 30 min. After washing with PBS for 10 min, the samples were reacted with avidin-biotin complex conjugated to horseradish peroxidase. The binding of antibodies was visualized with True Blue Peroxydase Substrate (Kirkegaard & Perry Lab, Gaithersburg, Md., U.S.A.)

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