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Partial characterization of collagen in several bivalve molluscs

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Abstract

Histological distribution and biochemical properties of collagen were examined in the edible tissues of five bivalve molluscan species. Collagen fibre was mainly distributed in the muscle connective tissues (epimysium, perimysium, and endomysium) of both mantles and adductors, and in the inner connective tissue matrix of the mantles. The collagen contents of the edible tissues varied considerably; the mantles showed much higher collagen content than the adductors. Soluble collagen could be recovered from the crude collagen fraction by a pepsin digestion method and was referred to as pepsin-solubilized collagen (PSC). The PSC showed a quite distinct SDS–PAGE pattern from those of collagen fractions prior to pepsin digestion for some species, suggesting considerable effects of pepsin digestion on the structure of the triple-helical domain.

Keywords: Collagen; Bivalve; Muscle; Mantle; Adductor; Connective tissue

1. Introduction

Collagen is a fundamental proteinaceous component in connective tissues of multicellular animals, playing mechanically and physiologically important roles in their bodies. It has recently been reported that collagen also functions as a significant factor for developing texture of raw or cooked meat from several aquatic animals (Mizuta, Yamada, Miyagi, & Yoshinaka, 1999; Mizuta, Yoshinaka, Sato, & Sakaguchi, 1997b; Olaechea, Ushio, Watabe, Takeda, & Hatae, 1993; Sato et al., 1997).

Bivalve mollusc belongs to commercially important groups of aquatic animals as a food resource, worldwide. As for collagen in bivalve molluscs, Pikkarainen et al. (1968) and DeVore, Engebretson, Schachtele, and Sauk (1984) reported the physicochemical and biochemical properties of collagen in the shell and byssus threads of blue mussel *Mytilus edulis*. Waite, Hansen, and Little (1989) isolated an adhesive protein with some features of collagen from the foot of the mussel, *Geukensia demissa*. Kimura, Nagaoka, and Kubota (1969) reported the properties of pepsin-solubilized collagen from the mantle of pearl oyster, *Pinctada martensii*. Suzuki, Yoshinaka, Mizuta, Funakoshi, and Wada (1991) reported the regeneration process of extracellular matrix, which consists mainly of collagen, during wound-healing of pearl oyster *Pinctada fucata* mantle, and partially characterized the pepsin-solubilized collagen using biochemical techniques. We recently characterized the collagen in the mantle and adductor of *P. fucata*, focussing on the structural change of its collagen during the solubilization process using pepsin (Mizuta, Miyagi, Nishimiya, & Yoshinaka, 2002). However, information is still limited on the biochemical or histochemical properties of muscle collagen in bivalve molluscs. In the present study, we tried to characterize collagens in edible tissues of several bivalve species, especially intact or non-pepsinized collagen, and to clarify the connective tissue configuration in their tissues to provide fundamental information concerning functions of collagen in the development of their texture.

2. Materials and methods

2.1. Preparation of collagens

Bivalve species used in the present study, listed in Table 1, were all obtained live. Three species, Japanese

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Table 1
Data of samples

Species	Month of sampling	Average body weight (g)	Sampled tissues
Dysodonta			
Isofilibranchia			
Blue mussel (*Mytilus galloprovincialis*)	July	33.4	Whole soft body
Purplish bifurcate mussel (*Septifer virgatus*)	April	9.7	Whole soft body
Ptericonchina			
Japanese scallop (*Patinopecten yessoensis*)	November	175.3	Adductor, mantle
Calloconchina			
Pacific oyster (*Crassostrea gigas*)	June	220.3	Adductor, mantle
Heterodonta			
Pliodontina			
Hard clam (*Meretrix lusoria*)	July	54.4	Adductor, mantle

scallop *Patinopecten yessoensis*, Pacific oyster *Crassostrea gigas*, and hard clam *Meretrix lusoria*, were obtained from local markets, while the other two species (blue mussel, *Mytilus galloprovoncialis* and purplish bifurcate mussel, *Septifer virgatus*) were obtained at the coast near Obama city. Edible tissues (mantle, adductor, or whole soft body) were dissected from the bodies, homogenized in 5 vol (v/w) of 0.1 M NaOH, and extracted for 24 h 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 washed thoroughly with distilled water, and extracted with 50 mM Tris–HCl, pH 7.5, containing 4 M guanidine hydrochloride (G/HCl) for 24 h at 5 °C, as previously described (Mizuta, Yoshinaka, Sato, & Sakaguchi, 1997a). The volume of the solvent used in the G/HCl extraction was adjusted to about one half (v/w) that of the initial tissue weight. After centrifugation at 10,000*g* for 20 min, the supernatant was dialyzed against distilled water overnight and then against 0.5 M acetic acid containing 2 M NaCl. The resultant precipitate was collected by centrifugation at 10,000*g* for 20 min, dialyzed against distilled water, and lyophilized. The preparation obtained was referred to as G/HCl-soluble collagen (GSC).

The insoluble matter, after the G/HCl extraction of the RS-AL, was washed thoroughly 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 collagen (PSC) preparation.

2.2. Chemical analysis

Protein nitrogen was determined by the micro-Kjeldahl method for insoluble material of tissues after extraction with 5% trichloroacetic acid. The factor 6.25 was used for converting the nitrogen content to protein content.

Collagen content was determined essentially as described previously (Mizuta, Yoshinaka, Sato, & Sakaguchi, 1994). The tissue was homogenized and extracted in 0.1 M NaOH according to the above method. After washing with distilled water, the residue obtained after the alkali extraction was further homogenized with a microhomogenizer (Polytron, Kinematica, Luzern, Switzerland) and then lyophilized. The lyophilized sample was hydrolyzed in 6 M HCl at 130 °C for 3.5 h. The hydroxyproline content in the hydrolysate was determined by the method of Woessner (1961). Collagen content was calculated from the hydroxyproline content, using the factor 10.23, which was determined from the hydrolysate of the PSC from the adductor of *P. fucata* (Mizuta et al., 2002).

2.3. SDS–PAGE analysis

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, of which 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 (Micko & Schlaepfer, 1978; Duhamel, 1983).

2.4. Histological observations

Histological observation with a light microscope was carried out essentially as described previously (Mizuta et al., 1994). The tissue was cut in to small pieces, fixed in Bouin's solution (Kiernan, 1990) for 6 h, and then embedded in paraffin (Parahisto, Nacalai Tesque, Kyoto, Japan). Sections (4 μm) were cut with a microtome. The prepared sections were stained with Azan stain (Kiernan, 1990) and examined with a light microscope (Optiphoto-2, Nikon, Tokyo, Japan).

3. Results and discussion

To examine the location of collagen fibre, the sections were prepared from the mantle and adductor of the four species, *M. galloprovincialis*, *P. yessoensis*, *C. gigas*, and *M. lusoria*, stained with Azan, and observed with the light microscope. Muscle and collagen fibres were stained orange red and blue, respectively. As shown in Fig. 1(a), (c), (e), and (g), the mantle tissues were relatively rich in connective tissue, showing subdermal (SDC) and inner (IC) connective tissues. In addition,

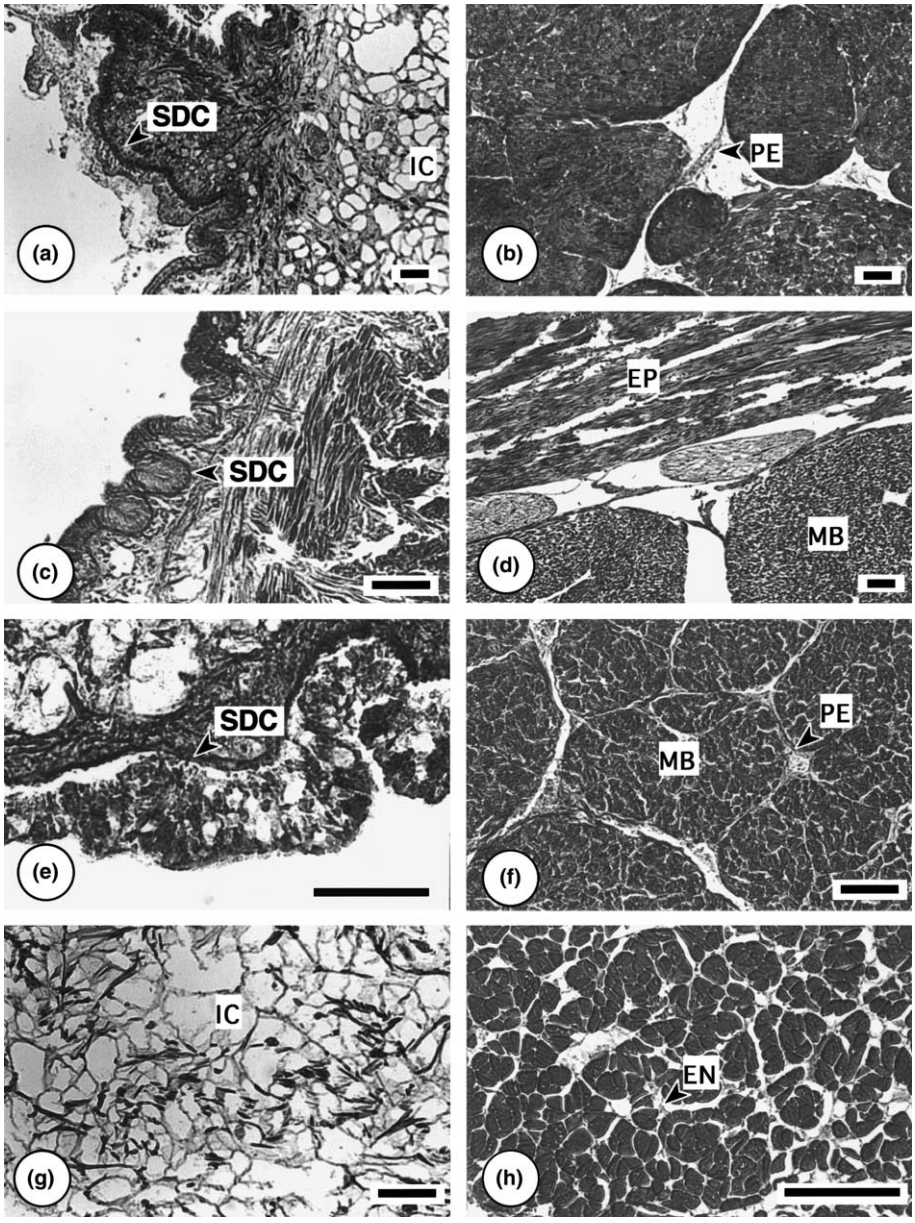

Fig. 1. Light micrographs of mantles ((a), (c), (e), and (g)) and adductors ((b), (d), (f), and (h)) of bivalve molluscan species stained with Azan. Muscle and collagen fibres were stained orange red and blue, respectively. (a) and (b), *Mytilus galloprovincialis*; (c) and (d), *Patinopecten yessoensis*; (e) and (f), *Crassostrea gigas*; (g) and (h), *Meretrix lusoria*. The letters SDC, IC, EP, PE, EN, and MB show subdermal connective tissue, inner connective tissue, epimysium, perimysium, endomysium, and muscle fibre bundle, respectively. Bars = 50 μm.

radial and longitudinal muscles were also observable in the mantles (data not shown). The adductors had thick connective tissue around muscle tissue (epimysium, EP), and thin connective tissue around muscle fibre bundle (perimysium, PE) and muscle fibre (endomysium, EN) (Fig. 1(b), (d), (f), and (h)). This type of connective tissue organization was also observed in the radial and longitudinal muscles in the mantle tissues (data not shown) and is very similar to those of *P. fucata* (Mizuta et al., 2002), vertebrate muscles (Bailey, Restall, Sims, & Duance, 1979; Light & Champion, 1984), and crustacean muscles (Mizuta, Yoshinaka, Sato, Suzuki, & Sakaguchi, 1992; Mizuta et al., 1994). In crustacean animals, it has been reported that thickness or distribution density of connective tissue is important for the formation of meat texture (Mizuta et al., 1994). Considering the similarity of connective tissue organization, it is necessary to investigate, in detail, the correlation between morphological characteristics of connective tissue and meat texture for bivalve molluscs.

The approximate collagen content in the edible tissues is shown in Table 2. As for the three species of *P. yessoensis*, *C. gigas*, and *M. lusoria*, comparison of collagen contents between tissues reveals higher values in the mantles than in the adductors. A similar result was also obtained for *P. fucata* in the previous study (Mizuta et al., 2002). Collagen content of these three species ranged from 0.75% to 1.19% of wet tissue and from 11.4% to 15.2% of protein for the mantles, and from 0.16% to 0.38% of wet tissue and from 1.3% to 2.8% of protein for the adductors, showing a considerable difference among species. In the case of two isofilibranchial species, *M. galloprovincialis* and *S. virgatus*, whole soft body was applied to the analysis, resulting in considerable differences, showing values of 0.20% and 0.83% of wet tissue, and 2.4% and 10.9% of protein, respectively. In gastropod species, the foot muscle of the abalone, *Haliotis discus*, was reported to show seasonal change of collagen content, corresponding well to the meat toughness (Olaechea et al., 1993). It is also important to investigate the seasonal change of collagen content in bivalve molluscan species to clarify the function of collagen in the development of meat texture.

The crude connective tissue fractions (RS-AL) from the tissues contained mucous material and were very insoluble in 0.5 M acetic acid, so we treated the RS-AL with G/HCl solution to remove the mucous material and solubilize part of the collagen. The G/HCl-soluble protein was effectively salted out by dialyzing against 0.5 M acetic acid containing 2 M NaCl, and consisted mainly of collagenous material. Approximately 2–3% of the total collagen was solubilized by this extraction method. On the other hand, about 10–30% of the total collagen could be solubilized from the residue, after the G/HCl extraction, by the limited pepsin digestion, for all the species examined.

Biochemical properties of the prepared collagen samples were partially examined by SDS–PAGE analysis (Fig. 2), compared with collagens from carp white muscle (Type I), and from the adductor of *P. fucata* prepared as reported previously (Mizuta et al., 2002). Considerable differences were observed in the SDS–PAGE patterns of the collagen preparations between species. For two of the species, of *M. galloprovincialis* and *P. yessoensis*, the SDS–PAGE patterns of RS-AL, GSC, and PSC were essentially similar to those of *P. fucata* (Mizuta et al., 2002). The RS-AL and GSC of these species showed two α chain-sized components, designated α1 and α2, on SDS–PAGE, with molecular weights estimated to be in the range 130–140 KDa, while the relative staining intensity of the α1 to α2 became much lower in PSC than in RS-AL or GSC, accompanied by the development of lower molecular weight components with metachromasy, as is the case with *P. fucata* (Mizuta et al., 2002). In the patterns of the RS-AL of *S. virgatus* and *C. gigas*, three α chain-sized components were observed, together with many lower molecular weight components, suggesting that some of the subunits might be unstable enough to be degraded during heat treatment in the presence of SDS. In contrast, the GSC of these species showed mainly two α chain-sized components with much fewer lower molecular weight components than RS-AL. The PSC from *S. virgatus* had a low relative staining intensity of the α1 to α2 chain and a higher level of low molecular weight components, whereas the SDS–PAGE pattern of the

Table 2
Collagen content of edible parts from the bivalve species

Species	Tissue	Collagen content	
		% of wet tissue	% of protein
Mytilus galloprovincialis	Whole soft body	0.20	2.4
Septifer virgatus	Whole soft body	0.83	10.9
Patinopecten yessoensis	Adductor	0.16	1.3
	Mantle	1.19	15.2
Crassostrea gigas	Adductor	0.38	2.8
	Mantle	0.75	11.4
Meretrix lusoria	Adductor	0.31	2.5
	Mantle	1.10	12.5

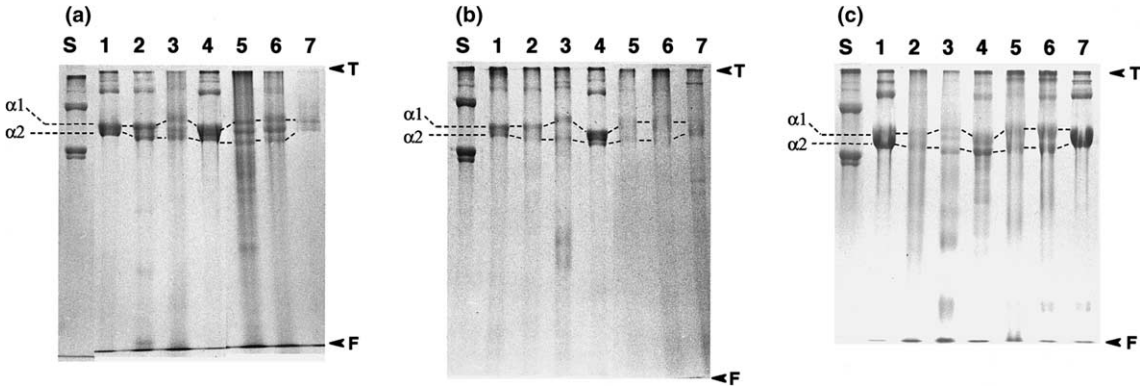

Fig. 2. SDS–PAGE analysis of RS-AL (a), guanidine hydrochloride-soluble collagens (b) and pepsin-solubilized collagens (c) from the tissues of several bivalve molluscs. Samples were resolved on 7.5% gels. Lane S, standard sample (carp Type 1 collagen); lane 1, adductor of *Pinctada fucata*; lanes 2 and 3, whole soft body from *Mytilus galloprovincialis* and *Septifer virgatus*, respectively; lane 4, adductor from *Patinopecten yessoensis*; lanes 5 and 6, adductor and mantle from *Crassostrea gigas*, respectively; lane 7, mantle from *Meretrix lusoria*. Arrowheads T and F show the top and buffer front of the gel, respectively.

PSC from *C. gigas* was similar to that of the GSC, showing less decrease in the relative staining intensity of the α1 to α2 chain. As for *M. lusoria*, the collagen preparations showed essentially similar behaviours on SDS–PAGE to those of *P. fucata*, *M. galloprovincialis*, and *P. yessoensis*, except for the occurrence of lower molecular weight components in the GSC. These combined results suggest that collagens of these five species essentially show two bands of α chains, α1 and α2, prior to pepsin digestion, and that the relative decrease of the α1 chain occurs conspicuously for the four species except for *C. gigas* by pepsin digestion.

Solubilization has been accepted as an important procedure for biochemical studies of collagen in tissues of aquatic animals. It has been reported that invertebrate collagens can be partially solubilized by limited pepsin digestion although they are highly insoluble (Kimura & Kubota, 1968; Kimura et al., 1969; Yoshinaka et al., 1990). In crustacean and molluscan species, the effects of pepsin digestion on each molecular species were examined for kuruma prawn, *Penaeus japonicus* (Mizuta et al., 1991; Yoshinaka, Mizuta, Suzuki, & Sato, 1991) and squid, *Todarodes pacificus* (Mizuta, Yoshinaka, Sato, & Sakaguchi, 1996; Mizuta et al., 1997a) by immunochemical techniques in extenso. Recently we reported structural changes of collagen in the *P. fucata* by limited pepsin digestion (Mizuta et al., 2002). In these species, the primary structures of specific collagen types or collagen subunits were fairly affected by pepsin digestion, accompanied by considerable changes in the SDS–PAGE patterns. However, information is still limited concerning the structural changes of collagen of bivalve molluscan tissues obtained by limited pepsin digestion. In the present study, the relative staining intensity of the α1 to α2 chain was apparently lower in PSC than in GSC or RS-AL for some species. In addition, considerable development of lower molecular weight components, some of which were considered to be degradation products of the α1 chain, was observed in the SDS–PAGE patterns of the PSC from some species. These combined results suggest the existence of pepsin-sensitive regions in the triple helical domain of the α1 chain, probably responsible for the degradation of the α1 chain and consequent development of lower molecular weight component during pepsin digestion. It is necessary to isolate the α1 chains from the GSC of bivalves and analyse their primary structures for determination of the pepsin-sensitive regions.

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