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Partial characterization of collagen in mantle and adductor of pearl oyster (*Pinctada fucata*)

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Abstract

Histochemical observation clarified the presence of collagen in the connective tissues of epimysium, perimysium, and endomysium in the mantle and adductor of the pearl oyster *Pinctada fucata*. A small amount of soluble collagen could be obtained from the mantle and adductor by extracting a crude connective tissue fraction with 4 M guanidine hydrochloride (G/HCl) solution, without protease digestion of telopeptides. The G/HCl-soluble collagen showed two alpha bands (α1 and α2) on SDS–PAGE. The relative staining intensities of the α1 to α2 chains were decreased gradually by pepsin digestion with concomitant development of lower molecular weight components. These results suggest the existence of pepsin-sensitive regions in the triple helical domain of the α1 chain.

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

1. Introduction

Muscle connective tissue of multicellular animals contains collagen as a fundamental proteinaceous component. It has recently been reported that muscle collagen is very important in the development of 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). In particular, minor collagens have received increasing 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 the 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).

Bivalve molluscs are accepted to be one of the commercially important groups of aquatic animals used as a food resource worldwide. As for collagen in bivalve molluscs, Pikkarainen, Rantanen, Vastamäki, Lampiaho, Kari, and Kulonen (1968) and DeVore, Engebretson, Schachtele, and Sauk (1984) reported the physicochemical and biochemical properties of collagen in the shell and byssus threads of sea mussel *Mytilus edulis,* respectively. 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*. This may be the first report concerning collagen in the muscle tissue of bivalve molluscs. However, little information is available on the biochemical characteristics of muscle collagen which is not pepsinized and is intact in primary structure. Suzuki, Yoshinaka, Mizuta, Funakoshi, and Wada (1991) reported the regeneration process of the extracellular matrix, which mainly consists of collagen, during wound healing of pearl oyster *Pinctada fucata* mantle, and partially characterized the pepsin-solubilized collagen using biochemical techniques. In Japan, pearl oyster is not only used as the mother shell in the production of pearl but is also important as a food resource. In the present study, we tried to characterize muscle collagen in the pearl oyster, especially intact or un-pepsinized collagen, and to clarify the connective

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tissue organization or distribution of the collagen fibre in muscular tissues in order to obtain fundamental information for elucidating the function of collagen in the development of meat texture.

2. Materials and methods

2.1. Preparation of collagens

Fifty individuals of cultured pearl oyster (average body weight, 58 g) were obtained alive from a local nursery at Mie Prefecture in January 1997. Muscle tissues (mantle and adductor, each of 500 g) were dissected out from the bodies, homogenized in five volumes (v/w) of 0.1 M NaOH, and extracted for 24 h with gentle stirring at 5 °C. The extraction was done to remove non-collagenous proteins and to prevent the effects 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 250 ml of 50 mM Tris–HCl, pH 7.5, containing 4 M guanidine hydrochloride (G/HCl) for 24 h at 5 °C as described previously (Mizuta, Yoshinaka, Sato, & Sakaguchi, 1997a). 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 (GS) collagen.

The insoluble matter remaining 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 (PS) collagen preparation.

2.2. Fractionation of PS collagen

The PS collagen was salted out, collected by centrifugation at 10,000 ***g*** for 20 min, and neutralized by washing with 50 mM Tris–HCl, pH 7.5, containing 4.4 M NaCl. Then the washed PS collagen was extracted with 2.4, 1.7 and 1.0 M NaCl, in succession. After each extraction the supernatant was recovered by centrifugation at 10,000 ***g*** for 20 min, and solid NaCl was added to the supernatants to final concentrations of 4.4, 2.4 and 1.7 M, respectively. The precipitated collagens were referred to as P-4.4, P-2.4 and P-l.7 fractions, while the final precipitate at 1.0 M NaCl extraction was referred to as the P-1.0 fraction.

2.3. Pepsin digestion of GS collagen

The GS collagen (100 μg) was suspended in 0.1 M acetic acid and digested with pepsin at an enzyme/substrate ratio (w/w) of 1:50 or 1:5 for 30 min or 3 h in a cold room at 5 °C. The digestion was terminated by addition of Tris and sodium dodecyl sulfate (SDS) to final concentrations of 0.125 M and 1%, respectively. The digests were resolved by SDS–polyacrylamide gel electrophoresis (PAGE).

2.4. Chemical analysis

Total nitrogen was determined by the micro-Kjeldahl method for the raw mantle and the adductor. Extractive nitrogen in the water-soluble fractions of mantle and adductor was also determined by the micro-Kjeldahl method after removal of protein components with 5% trichloroacetic acid. Protein nitrogen was calculated by subtracting the extractive nitrogen from the total nitrogen. The factor of 6.25 was used for converting the nitrogen content to protein content.

The collagen content was determined essentially as decribed previously (Mizuta, Yoshinaka, Sato, & Sakaguchi, 1994a). The muscle 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). The factor of 10.23, which had been determined from the hydrolysate of the PS collagen from the adductor, was used for converting the hydroxyproline content to collagen content.

2.5. SDS–PAGE analysis

SDS–PAGE was performed by the method of Laemmli (1970), using 5 or 7.5% polyacrylamide gels. The samples (about 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 (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) was performed essentially as described by Cleaveland, Fischer, Kirshner, and Laemmli (1977). The sample (about 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.

2.6. Histological observations

Histological observation with a light microscope was carried out essentially as described previously (Mizuta et al., 1994a). The muscle tissue was cut into 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

It is very important to clarify the distribution of collagen fibre or connective tissue in mantle and adductor in order to discuss or elucidate the contribution of collagen to the development of meat texture. To examine the location of collagen fibre, the prepared sections were stained with Azan stain. Muscle and collagen fibres were stained orange red and blue, respectively. As shown in Fig. 1a, thick connective tissue was observed at the subdermal layer (subdermal connective tissue, SDC), and radial muscle (RM) located at the inner part of the mantle. The rest of the inner mantle was very rich in connective tissue (Fig. 1a and c; inner connective tissue, IC). The magnified images of the mantle radial muscle and adductor are shown in Fig. 1b–1f, exhibiting thick connective tissue around muscle tissue (epimysium, EP), and thin connective tissue around muscle fibre bundle (perimysium, PE) and muscle fibre (endomysium, EN). This type of connective tissue organization is very similar to those of vertebrate muscle (Bailey, Restall, Sims, & Duance, 1979; Light & Champion, 1984) and crustacean muscle (Mizuta et al., 1994a; Mizuta, Yoshinaka, Sato, Suzuki, & Sakaguchi, 1992). Garcia-Gasca, Ochoa-Baez, and Betancourt (1994) examined the microstructure of mantle of the pearl oyster *Pinctada mazatlanica* in detail, showing the presence of four types of muscle, which are radial, longitudinal, oblique, and transversal muscles. In the present study, similar connective tissue organization was also observed in the longitudinal muscle (data not shown) although it was very difficult to observe or identify the latter two types. In addition, Mikhailov, Torrado, Méndez, and López (1996) also described the presence of perimysium and endomysium in the adductor of the mussel *Mytilus gauoprovincialis* showing, the tissue distribution of "mantle connective tissue polypeptide" by immunohistochemical techniques.

Approximate collagen contents in the mantle and adductor were 1.03 and 0.66% of wet tissue, and 19.2 and 4.6% of protein, respectively. The collagen content per unit weight protein of the mantle was about four times as much as that of the adductor, and the content per wet tissue was about 1.5 times. These results corresponded well to the visual difference in the connective tissue structure or density between the mantle and adductor as shown in Fig. 1. As the crude connective tissue fractions (RS-AL) from the mantle and adductor were quite insoluble in 0.5 M acetic acid, we tried to solubilize a part of collagen by extracting the RS-AL with G/HCl solution. The collagenous component in the G/HCl-soluble fractions was almost completely salted out by dialyzing against 0.5 M acetic acid containing 2 M NaCl, and was shown to consist mainly of a collagenous component by estimating the hydroxyproline in them. Approximately 2–3% of the total collagen was solubilized by this extraction method for both mantle and adductor. SDS–PAGE patterns of the GS collagens are shown in Fig. 2A, where they are compared with Type I collagen from carp white muscle. The GS collagens from the mantle and adductor showed similar SDS–PAGE patterns to each other, exhibiting two α chain-sized components (α1 and α2) and one β chain-sized component (β) with slower mobility than those of the corresponding components in carp Type I collagen. When compared with α1(I) and β(I) chains, with approximate molecular weights of 100 and 200 kDa, respectively, the molecular weight of the α1 and α2 chains was estimated to be about 130–140 kDa. All these chains showed metachromasy, characteristic of collagenous components. In peptide mapping, these GS collagens showed similar patterns (Fig. 2B), indicating a close similarity of compositional collagen types between connective tissues of mantle and adductor.

The GS collagen from the adductor was digested with pepsin to examine the effect of pepsin digestion on it (Fig. 2C). The staining intensity of the α1 chain and other higher molecular weight components was reduced by pepsin digestion with concomitant development of

lower molecular weight components, designated by an asterisk, while the α2 chain appeared not to be affected. Approximately 10–20% of the insoluble matter after the G/HCl extraction could be solubilized by limited pepsin digestion, and the rest remained insoluble. The PS collagens from the mantle and adductor in the present study showed similar SDS–PAGE patterns to each other, and to that of the pepsin digest of the GS collagen from the adductor (Fig. 3A). Relative staining intensity of the α1 to α2 chains was about 1.6 for the GS collagen but about 0.6 for the PS collagen when estimated by the image analysis system. The PS collagen from the adductor was fractionated by differential salt precipitation to obtain information concerning the compositional molecular species (Fig. 3B). The four fractions obtained (P-4.4, P-2.4, P-1.7 and P-1.0) showed quite similar SDS–PAGE patterns to one another, and the addition of 2-mercaptoethanol did not affect the SDS–PAGE patterns, suggesting the absence of molecular species with disulfide bonds. In several fish (Sato, Yoshinaka, Itoh, & Sato, 1989) and squid (Mizuta, Mutoh, Sugihara, & Yoshinaka, 2000; Mizuta, Yoshinaka, Sato, & Sakaguchi, 1994b) species, it has been reported that minor collagen types can be recovered in the Type V collagen fraction (P-4.4 fraction), while the present experiment failed to concentrate a minor collagen type in any fraction. Moreover, we could not find collagenous bands corresponding to Type V collagen in the SDS–PAGE patterns of the GS and PS collagens. It can be considered that mantle and adductor of pearl oyster may contain little Type V-like collagen or that the content of this minor type may be much less than those of fish or squids.

Limited pepsin digestion has been adopted as a very powerful method to solubilize invertebrate collagens which are highly insoluble (Kimura & Kubota, 1968;

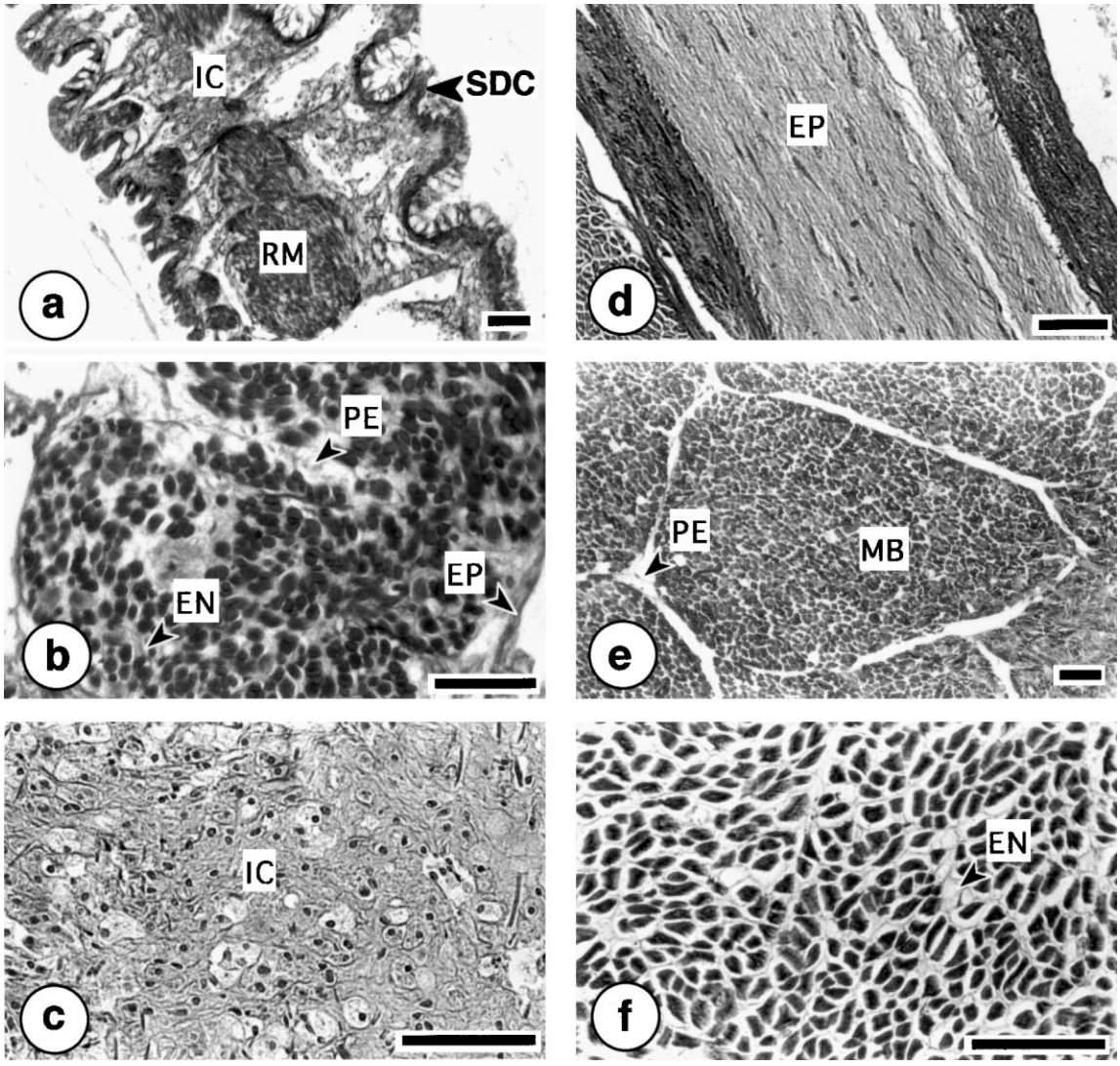

Fig. 1. Light micrographs of mantle (a, b and c) and adductor (d, e and 1) tissues of the pearl oyster stained with Azan stain. Muscle and collagen fibres were stained orange red and blue, respectively. The letters SDC, RM, IC, EP, PE, EN and MB show subdermal connective tissue, radial muscle, inner connective tissue, epimysium, perimysium, endomysium, and muscle bundle, respectively. Bars = 50 μm.

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, Yoshinaka, Sato, Suzuki, Itoh, & Sakaguchi, 1991; Yoshinaka, Mizuta, Suzuki, & Sato, 1991) and squid *Todarodes pacificus* (Mizuta, Yoshinaka, Sato, & Sakaguchi, 1996; Mizuta et al., 1997a) by immunochemical techniques in detail. However, little information is available concerning the structural change of collagen of bivalve tissues by limited pepsin digestion. In the present study, the relative proportion of the α1 to α2 chains of the GS collagen was shown to decrease by pepsin digestion with concomitant development of lower molecular weight components. In addition, the PS collagen also exhibited quite a similar pattern to those of the pepsin digests of the GS collagen. In our preliminary experiment, we prepared PS collagen from the RS-AL without G/HCl treatment considering for some effect of G/HCl treatment on collagen, but the same result was obtained as the PS collagen from G/HCl-treated RS-AL (data not shown). These combined results suggest that existence of pepsin-sensitive regions in the triple helical domain of the α1 chain may result in the degradation of the α1 chain and consequent development of lower molecular weight component in pepsin digestion. It seems to be

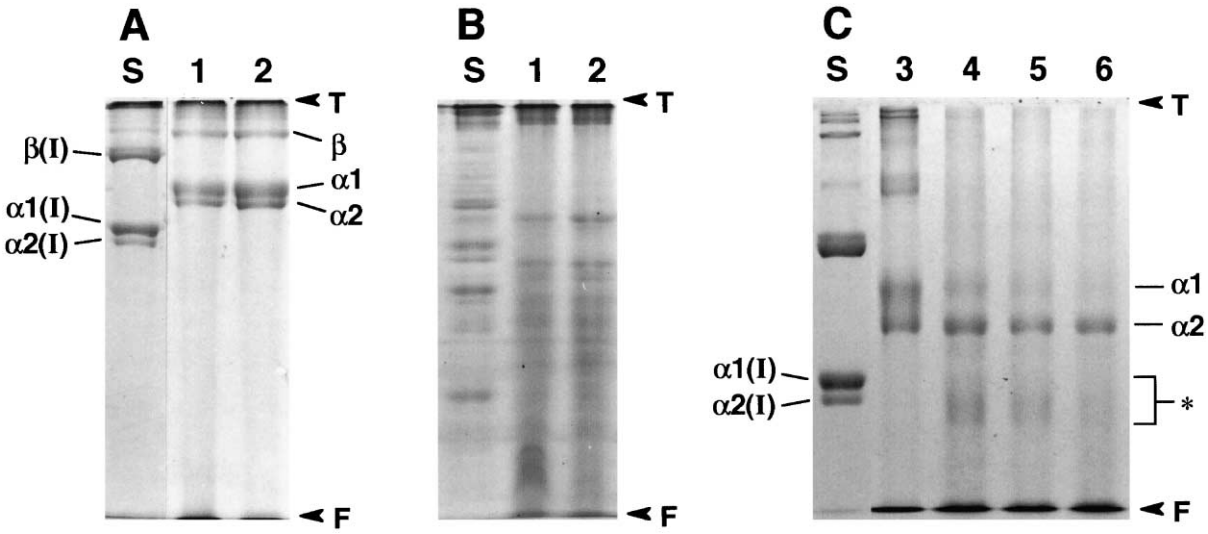

Fig. 2. SDS–PAGE analysis (A and C) and V-8 protease peptide maps (B) of guanidine hydrochloride-soluble (GS) collagen or its pepsin digests from the mantle (lane 1) or adductor (lanes 2–6) of the pearl oyster. Samples were resolved on 7.5% (A), 12.5% (B), or 5% (C) gel. Lane S, standard sample (carp Type I collagen); lanes 1–3, GS collagen; lanes 4–6, GS collagen digested with 1/50 pepsin for 30 min, 1/50 pepsin for 3 h, and 1/5 pepsin for 30 min, respectively. Arrowheads T and F show the top and buffer front of the gel, respectively.

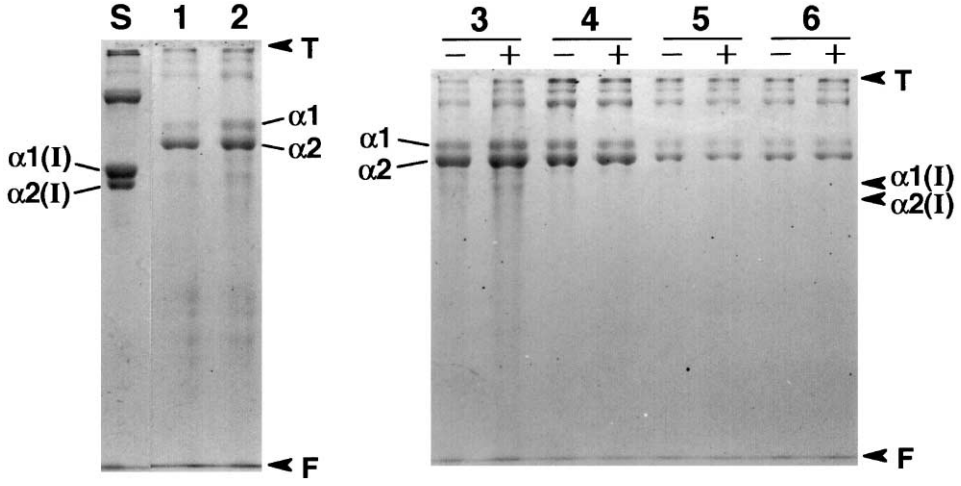

Fig. 3. SDS–PAGE analysis (7.5% gel) of pepsin-solubilized (PS) collagens from the mantle (lane 1) and adductor (lane 2) and fractions obtained after differential salt precipitation using NaCl (lanes 3–6). Lane S, as in Fig. 2; lanes 3–6, P-4.4, P-2.4, P-1.7 and P-1.0 fractions, respectively. Symbols (+) or (−), with or without 2-mercaptoethanol reduction, respectively. Arrowheads α1(I) and α2(I) show the positions of α1(I) and α2(I) chains, respectively. Arrowheads T and F, as in Fig. 2.

needed to purify the α1 chain from the GS collagen or to search for other bivalve species possessing collagen which is stable to pepsin digestion for detailed investigation on biochemical properties of bivalve collagen. Further studies are now in progress on the effect of protease digestion on the collagen molecule in bivalve muscle using immunochemical and biochemical techniques.

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