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Comparison of collagen types of arm and mantle muscles of the common octopus (*Octopus vulgaris*)

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Abstract

Properties of muscle collagens of arm and mantle of the common octopus, *Octopus vulgaris*, were compared with regard to molecular species and thermal behaviour. The major collagens were isolated from pepsin-solubilized collagen preparations of these tissues by salt precipitation and were shown, by peptide mapping, to be essentially identical to each other in the triple helical domain. Alpha and beta subunits of the major collagen of the arm, in intact form (not solubilized by pepsin digestion), on electrophoresis, moved more slowly than those of the mantle. The major collagen of the arm showed higher hot-water solubility and lower thermal stability than that from the mantle muscle. These results suggested that there were some structural and functional differences between the major collagens from these tissues although they were genetically identical molecular species. In addition, immunoblot analysis revealed the existence of a minor collagen type, which corresponded to Type SQ-II collagen in decapod molluscs, in both arm and mantle muscles.

Keywords: Collagen; Octopus; Cephalopod; Mollusc; Mantle; Arm; Connective tissue

1. Introduction

Collagen is an essential component of muscle connective tissues in multicellular animals. Its importance in expression of texture of raw or cooked meat has been suggested for several aquatic animals (Mizuta, Yamada, Miyagi, & Yoshinaka, 1999; Mizuta, Yoshinaka, Sato, & Sakaguchi, 1994a, 1997b; Olaechea, Ushio, Watabe, Takeda, & Hatae, 1993; Sato, Yoshinaka, Sato, & Shimizu, 1986). Quantitatively minor collagens, especially, have received increasing attention as critical components affecting post mortem textural change of fish meat during chilled storage (Sato et al., 1997).

Cephalopod molluscs are accepted as being commercially important aquatic animals that are used as a food resource. The common octopus *Octopus vulgaris* is the main species in octopodan animals, from which meat is preferably eaten either cooked or raw, in Japan. As for collagen in octopod molluscs, Kimura, Takema, and Kubota (1981) demonstrated that the major collagen type in the skin of the common octopus was a heterotrimer $(\alpha1)_2\alpha2$. Takema and Kimura (1982) described the existence of two genetically distinct types of collagen in the arm muscle of the common octopus. Babu and Chandrakasan (1984) isolated and characterized the major collagen in the suckers of an octopodan species as a heterotrimer $(\alpha1)_2\alpha2$. However, little information is available about the properties of "intact" collagen, which is not solubilized and intact in primary structure, in octopus muscle tissues. In the present study, we tried to partially characterize the collagen types in the arm and mantle muscles by biochemical techniques and to compare thermal behaviours between the major collagens from the arm and mantle muscles as a basis for the study of textural development in raw or cooked meat.

2. Materials and methods

2.1. Preparation and fractionation of collagens

Common octopus *O. vulgaris* (five individuals; average body weight, 1020 g) were obtained alive from a

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local market in April. Muscle tissues (about 500 g) were dissected out from the arm and mantle, homogenized in 5 volumes (v/w) of 0.1M NaOH, and extracted in the same solution for 24 h with gentle stirring 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, Sato, Sato, Itoh, Hujita, & Ikeda, 1985). The residue, after alkali extraction (RS-AL), was thoroughly washed with distilled water, and extracted with 250 ml of 50 mM Tris–HCl, pH7.5, containing 4 M guanidine hydrochloride (G/HCl) for 24 h at 5 °C, as described previously (Mizuta et al., 1997a). This extraction was done to solubilize the part of collagen with intact primary structure without pepsin digestion and to enhance the yield of pepsin-solubilized collagen described below. 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; crystallized and lyophilized; Sigma, St. Louis, MO, USA) 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 0.5M acetic acid containing 0.45 M NaCl. The soluble and precipitable fractions were separated from each other by centrifugation at 10,000 *g* for 20 min, and referred to as S-0.45 and P-0.45 fractions, respectively.

2.2. SDS (sodium dodecyl sulphate) – PAGE (polyacrylamide gel electrophoresis)

SDS–PAGE was performed by the method of Laemmli (1970) using 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 a 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).

2.3. Peptide mapping

Peptide mapping with glutamyl endopeptidase from *Staphylococcus aureus,* strain V-8 (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 Cleveland, 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.

2.4. Hotwater solubility of collagens

Hotwater solubility was measured as reported previously (Mizuta et al., 1999). The RS-AL was suspended in 20 mM sodium phosphate, pH 7.2, at a concentration of 1.0 mg/ml and homogenized at 500 *g* for 2 min by a microhomogenizer (Polytron; Kinematica, Luzern, Switzerland). The homogenate was heated at 20, 30, 40, 50, 60, 70, 80, and 90 °C for 30 min in a water bath (NCB-3200, Tokyo Rikakikai, Tokyo, Japan) and centrifuged at 12,000 *g* for 20 min. Hydroxyproline in the supernatant was determined by the method of Woessner (1961). The total hydroxyproline content was predetermined, and the hotwater solubility (%) of the octopus collagen was expressed as the relative proportion of the hydroxyproline in the hotwater-soluble fraction to the total hydroxyproline. Some soluble fractions were also examined by SDS–PAGE.

2.5. Thermal stability of collagens

Thermal stability was compared for collagens in the RS-AL from the arm and mantle muscles by monitoring the protease-induced degradation of helical domains by SDS–PAGE. The RS-AL was suspended in 0.1 M acetic acid at a concentration of 4 mg/ml, and homogenized at 500 *g* for 2 min by a microhomogenizer (Polytron). The homogenate was heated for 30 min at appropriate

temperatures ranging from 20 to 42.5 °C and digested with pepsin at an enzyme/substrate ratio of 1:10 for 20 h at 5 °C. The digestion was terminated by adding SDS, Tris, and urea to final concentrations of 1%, 0.125, and 2 M, respectively. The digest at each temperature was analyzed by SDS–PAGE.

2.6. Immunoblotting

After separation by SDS–PAGE, collagen chains were transferred to nitrocellulose membranes according to Towbin, Staehelin, and Gordon (1979), and the membrane was immunostained with an antiserum by the avidin–biotin complex method. The membrane was blocked in 2% hen egg ovalbumin in phosphate buffered saline (PBS; 10 mM sodium phosphate, pH 7.4, containing 0.15 M NaCl) and then reacted with the antiserum diluted 1/1000–1/100 in PBS. Biotinylated anti-rabbit IgG was diluted 1/500–1/200 in PBS and used as the secondary antibody. After the membrane was reacted with the avidin–biotin complex, the binding of antibody was visualized using 0.04% diaminobenzidine tetrahydrochloride (Sigma) or 0.04% 4-chloro-1-naphthol (Nacalai Tesque, Kyoto, Japan) containing 0.006% H_2O_2.

2.7. Chemical analysis

Protein nitrogen was determined by the micro-Kjeldahl method (Van Camp & Huyghebaert, 1996) for insoluble material of muscle after extraction with 5% trichloroacetic acid. The value of 6.25 was used as the factor for converting the nitrogen content to protein content.

Collagen content was calculated from the value of hydroxyproline which was determined by the method of Woessner (1961). The value of 11.1, which had been determined from the hydrolysate of the PS collagen preparation from the mantle muscle of squid, *Todarodes pacificus* (Mizuta, Yoshinaka, Sato, & Sakaguchi, 1994b), was used as the factor for converting the hydroxyproline content to collagen content.

3. Results and discussion

The approximate collagen contents in the present samples (arm and mantle muscles) were 1.4 and 1.9% of wet tissue, and 9.1 and 14% of protein, respectively. Crude collagen was successfully recovered as RS-AL preparations from the arm and mantle muscles by the extraction with 0.1 M NaOH. Although the collagens from the arm and mantle muscles were insoluble in 0.5 M acetic acid, approximately 9.0 and 16% of the total collagen was soluble in 4 M G/HCl solution, respectively. On the other hand, more than 80% of the total collagen could be solubilized for both tissues by pepsin digestion. The RS-AL, GSC, and PSC from the mantle muscle showed similar SDS–PAGE patterns to one another, showing two major α chains, designated as α1 and α2, along with β chain-sized components and even higher molecular weight components (Fig. 1A, Mantle). These results indicate that the structure of the major collagen in the mantle muscle is not much affected by the extraction with G/HCl or by digestion with pepsin. In contrast, the α and β subunits in the RS-AL from the arm muscle showed slower mobility than those of the other two collagen preparations from the arm muscle and of the three collagen preparations from the mantle muscle. (Fig. 1A, Arm). These results suggest that the structure of the major collagen from the arm muscle

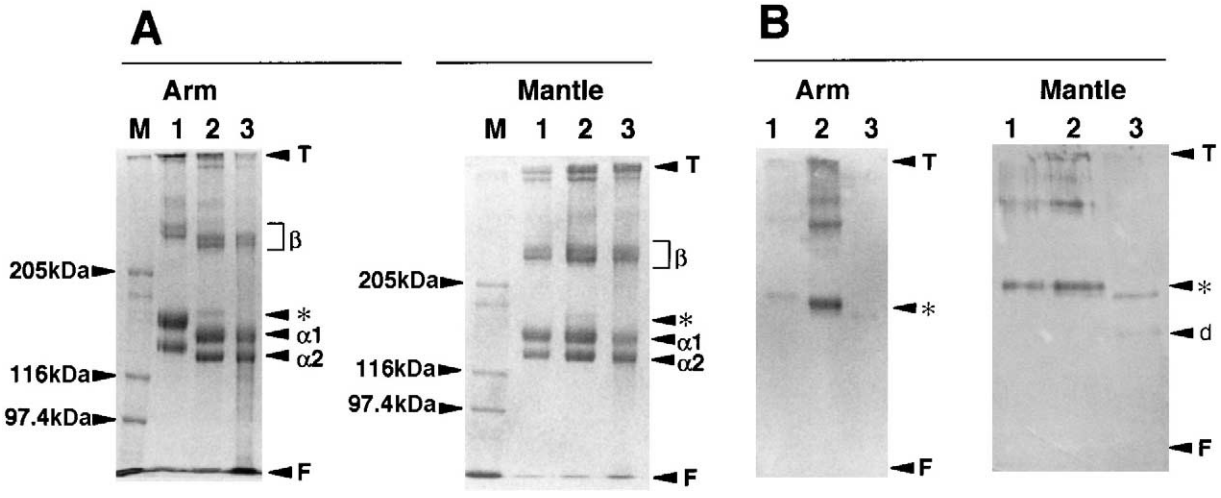

Fig. 1. SDS–PAGE (A) and immunoblot analysis (B) of the RS-AL (1), G/HCl-soluble collagens (2), and pepsin-solubilized collagens (3) from the arm (Arm) and mantle (Mantle) muscles of the octopus. The molecular weight standard shows three bands of myosin from rabbit muscle (205 K), β-galactosidase from *Escherichia coli* (116 K), and phosphorylase b from rabbit muscle (97.4 K). Arrowheads T and F show the gel top and buffer front, respectively.

may be affected by the extraction with G/HCl or digestion with pepsin. Possibly the major collagen may possess some adhering materials which can be removed by the extraction with G/HCl solution, however, details of its structural change remained to be solved.

On the other hand, the GSC from both arm and mantle muscles showed a minor band at the position indicated by an asterisk (*), of which mobility is comparable to that of the α1(SQ-II) chain of the minor collagen in squid mantle and skin (Type SQ-II; Mizuta et al., 1994b). Fig. 1B shows the result of immunoblot analysis carried out for the RS-AL, GSC, and PSC from both arm and mantle muscles using anti-α1(SQ-II) component serum prepared previously (Mizuta, Yoshinaka, Sato, & Sakaguchi, 1995). Positive bands were observed for the RS-AL and GSC at the position of the asterisk along with the bands with higher molecular weights, and for the PSC slightly below the position of the asterisk. Moreover, a faint positive band was detected below the position of the asterisk, indicated by the arrowhead **d**, in the immunoblot pattern of the PSC from the mantle muscle, suggesting degradation products from the possible breakdown of the triple-helical region of this minor collagen type by pepsin digestion. These results suggest the presence of a minor molecular species corresponding to Type SQ-II collagen of squid muscle and skin (Mizuta et al., 1994b) and the structural changes of this minor collagen type by pepsin digestion. In the previous paper (Mizuta et al., 1997a), we reported that the minor collagen (Type SQ-II) in the mantle muscle of squid *Todarodes pacificus* could be preferably solubilized from RS-AL by extraction with 4 M G/HCl solution. The minor collagen in the arm and mantle muscles of the octopus seemed to be slightly concentrated in the GSC preparations because of the higher relative reactivity of the positive components to the anti-α1(SQ-II) serum in immunoblot analysis than that in the RS-AL. However, it was very difficult to concentrate the minor collagen type by G/HCl extraction as much as that of squid, due to the differences in solubilities of the major and minor collagen types of these species.

The PSC was fractionated by differential salt precipitation with 0.5 M acetic acid containing 0.45 M NaCl to separate the minor molecular species from the major one, based on the previous result for squid muscle and skin (Mizuta et al., 1994b). Almost all of the PSC, however, precipitated in this condition, and the fractionation of the minor molecular species was not successful, probably due to its quantitative decrease with degradation of the triple helical region by pepsin digestion. The precipitable fractions, at 0.45 M NaCl (P-0.45 fractions), were analyzed by SDS–PAGE and peptide mapping, along with Type SQ-I and SQ-II collagens from the squid mantle muscle (Fig. 2). The SDS–PAGE patterns of the P-0.45 fractions were essentially identical to the PSC and were similar to Type SQ-I collagen from squid, but were distinct from Type SQ-II collagen (Fig. 2A). The P-0.45 fractions from the arm and mantle muscles showed identical patterns to each other in peptide mapping with both glutamyl endopeptidase (Fig. 2B) and lysyl endopeptidase (Fig. 2C), suggesting genetical identity between the major collagens from the arm and mantle muscles. Incidentally, the major collagens in the octopus muscle showed little resemblance to the squid collagens, Type SQ-I and SQ-II, in peptide maps.

Takema and Kimura (1982) reported the presence of a minor collagen type in the octopus arm muscle, which had disulfide bonds and showed mainly a γ chain sized component on SDS–PAGE in the absence of reducing agents, e.g. dithiothreitol. However, the minor collagen type found in the present study represented mainly α and β chains, and a trace amount of γ chain on immunoblot

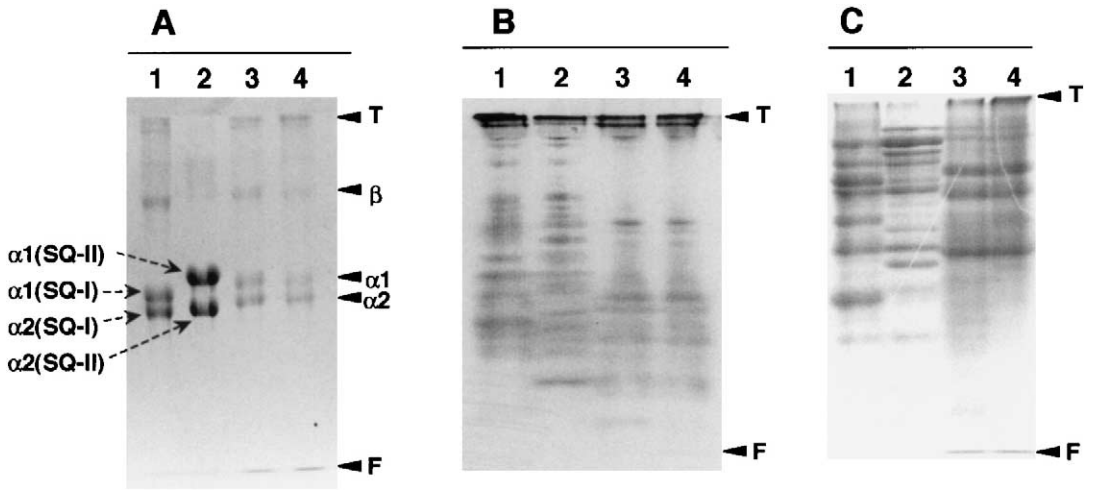

Fig. 2. SDS–PAGE analysis (A) and peptide mapping with glutamyl endopeptidase (B, 12.5% gel) and lysyl endopeptidase (C, 10% gel) of Type SQ-I (1) and SQ-II (2) collagens from the squid mantle muscle and the P-0.45 fractions from the arm (3) and mantle (4) muscles of the octopus.

analysis using antiserum against α1 component of Type SQ-II collagen from the squid mantle muscle. These combined facts suggest that the minor collgen type found in the present study should not correspond to the minor collagen type with disulfide bonds reported by Takema and Kimura (1982) but to another collagen type which is similar to the squid minor collagen, Type SQ-II. Although isolation of the minor collagen type was not successful in the present study, further study is in progress to clarify the properties of this molecule.

Hotwater solubility was measured for collagens in fibre form (RS-AL) from the arm and mantle muscles (Fig. 3). At any temperature adopted, the hotwater solubility of the collagen from the arm muscle showed constantly higher values than the collagen from the mantle muscle. The arm muscle collagen had hotwater solubility of about 20–40% from 30 to 60 °C, showing no pronounced difference from the mantle muscle collagen. A rapid increase, however, was observed from 70 °C, and the solubility reached more than 90% at 80 and 90 °C, showing a marked difference from the mantle muscle collagen. The soluble fractions at 60–90 °C of the RS-AL from the arm and mantle muscles exhibited almost identical SDS–PAGE patterns to those of the RS-AL shown in Fig. 1, confirming the existence of collagen subunits in the soluble fractions. In Fig. 4, thermal stability, expressed as resistance to protease digestion, is compared between the collagens from the two tissues. The relative staining intensity of the α components (α1 and α2) of the major collagen decreased rapidly from 22.5 to 27.5 °C for the arm, and from 27.5 to 32.5 °C for the mantle. These combined results indicate that there are apparent differences in the thermal properties between the major collagens from the arm and mantle muscles. Further detailed studies, however, appear to be needed concerning variation of thermal behaviour of collagens in these muscle tissues with sampling season, and species.

In the present study, genetic identity of the triple helical domain is suggested for the major collagens in the arm and mantle muscles. On the other hand, the present SDS–PAGE analysis indicates that the structure of the arm major collagen might be modified by G/HCl treatment or pepsin digestion, while little effect of these treatments was observed for the collagen from the mantle muscle. Such qualitative differences suggest that collagen may have distinct functions to develop the textural characteristics of arm and mantle muscles. Further

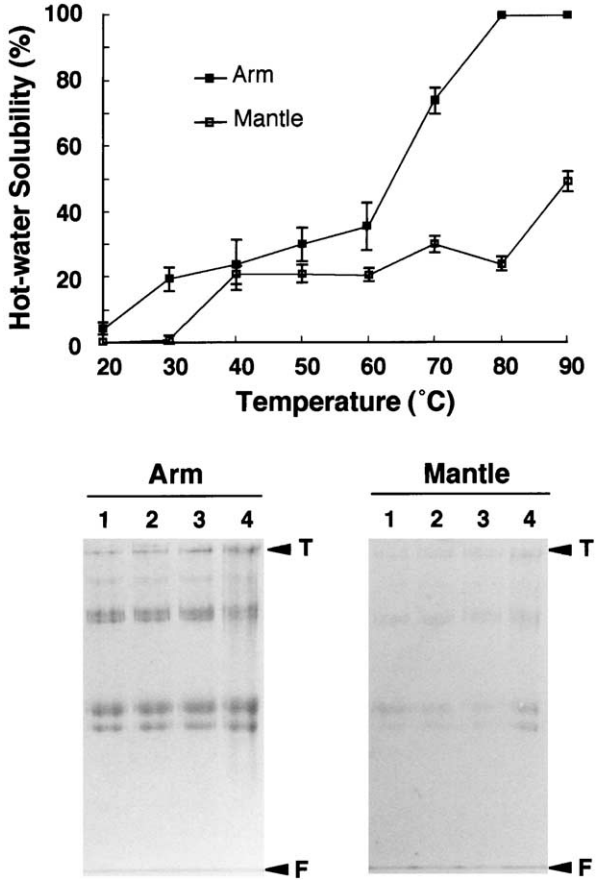

Fig. 3. Hotwater solubility of the collagens from the octopus arm (Arm) and mantle (Mantle) muscles, along with SDS–PAGE patterns of the soluble fractions at 60 (1), 70 (2), 80 (3), and 90 °C (4).

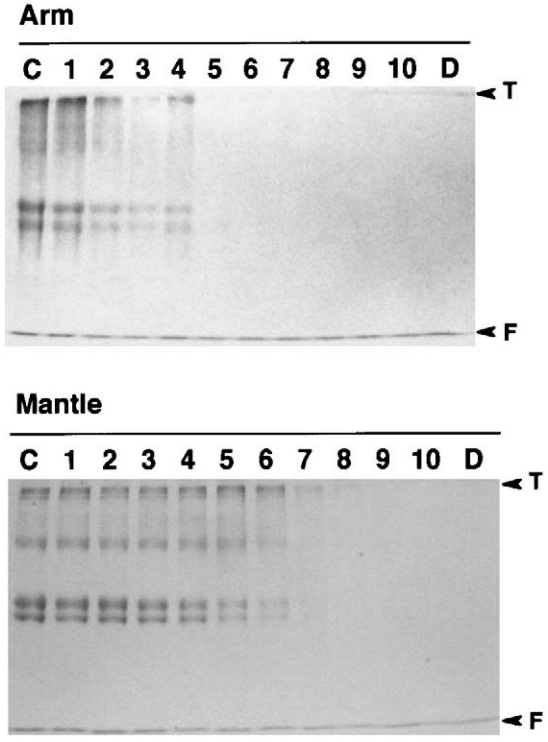

Fig. 4. Comparison of thermal stability of the major collagens from the octopus arm (Arm) and mantle (Mantle) muscles by monitoring the protease-induced degradation of helical domains by SDS–PAGE. The RS-AL were suspended in 0.1 M acetic acid, and incubated at 20 (lane 1), 22.5 (lane 2), 25 (lane 3), 27.5 (lane 4), 30 (lane 5), 32.5 (lane 6), 35 (lane 7), 37.5 (lane 8), 40 (lane 9), and 42.5 °C (lane 10). Lanes C and D, respectively, show the control (unheated) and the sample which was completely denatured by heating at 80 °C for 10 min.

studies are now in progress to elucidate the differences in structure of the major collagens in arm and mantle muscles in relation to their thermal differences and textural properties.

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