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Characterization of molecular species of collagen in muscles of Japanese amberjack, *Seriola quinqueradiata*

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Abstract

Pepsin-solubilized collagens prepared from the muscle tissues (ordinary and dark muscles) of Japanese amberjack were separated into two fractions, major and minor, by ammonium sulphate precipitation. Collagens in these fractions were further purified by cation-exchange column chromatography. The results of SDS-PAGE, peptide mapping, and amino acid analysis suggested that the purified major and minor collagens might be classified as type I and V collagens, respectively. Each type of collagen was fundamentally similar, among the ordinary and dark muscles, in amino acid compositions and peptide maps.

Keywords: Fish; Muscle; Type I; Type V; Molecular species; Connective tissue

1. Introduction

Collagen, as the major constituent of fish connective tissue, has been demonstrated to exist in different genetic forms. In ordinary muscle and skin of fish, at least two types of collagen, type I and V collagens, were found to be major and minor collagens, respectively (Kimura, Ohno, Miyauchi, & Uchida, 1987; Kimura, Zhu, Matsui, Shijoh, & Takamizawa, 1988; Sato, Yoshinaka, Sato, Itoh, & Shimizu, 1988; Sato, Yoshinaka, & Sato, 1989; Yata, Yoshida, Fujisawa, Mizuta, & Yoshinaka, 2001). Collagen was reported to be related to raw fish meat texture: the higher the collagen content, the firmer was the meat (Hatae, Tobimatsu, Takeyama, & Matsumoto, 1986; Sato, Yoshinaka, Sato, & Shimizu, 1986). Fish muscle tenderization during post-mortem aging has been studied (Ando, Toyohara, Shimizu, & Sakaguchi, 1993; Toyohara & Shimizu, 1988). Recently, enzymatic degradation of type V collagen was found to be responsible to its tenderization (Sato et al., 1997). Fish have the unique tissue called superficial dark muscle, which lies under the lateral line. Some fish have the deep-seated dark muscle, along with the superficial one described above. However, there is scant information about collagen in dark muscle (Kanoh et al., 1986; Zhu & Kimura, 1999). It is necessary to obtain information about dark muscle collagen to study textural changes of dark muscle during post-mortem aging or heat-treatment. Japanese amberjack, *Seriola quinqueradiata*, is a popular red-fleshed fish and is one of the important fish stocks which are consumed widely in Japan. The dark muscle of this species amounts to 14% of whole muscle (Fujikawa & Naganuma, 1936). In this paper, we tried to classify molecular species of collagen in the ordinary and dark muscle of Japanese amberjack.

2. Materials and methods

2.1. Materials

Fresh individuals of Japanese amberjack, *Seriola quinqueradiata* (average body weight 1.14 kg), which had been just landed, were obtained from a local fish market in Obama city. Pepsin (crystallized and lyophilized, EC 3.4.23.1) was obtained from Sigma Chemical Co. (St.Louis, MO, USA). All other reagents were of analytical grade.

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2.2. Preparation of collagens

All procedures were performed in a cold room at 5 °C. The fish were skinned and filleted. The dark and ordinary muscles were separated from fillet. Each muscle tissue (about 300–500 g) was homogenized with 3 vol. (v/w) of 0.1 M NaOH, using a non-bubbling homogenizer (NS-2; Nissei, Tokyo, Japan). The suspensions were stirred overnight and centrifuged at 10,000×*g* for 20 min. To the residues, 10 vol. of 0.1 M NaOH were added and the suspensions were stirred overnight. This procedure was repeated three or four times. The residue after alkali-extraction (RS-AL) was washed thoroughly with distilled water and then suspended in 0.5 M acetic acid (pH 2.5). The RS-AL of each muscle was digested with pepsin, to cleave the non-helical region of collagen, telopeptide, at an enzyme/substrate ratio of 1/20 to 1/40 (w/w), for 24 h at 5 °C. The collagen in the resultant supernatant after centrifugation was salted out by adding NaCl to give a final concentration of 2.0 M. After centrifugation at 10,000×*g* for 20 min, the resultant precipitate was used as a pepsin-solubilized collagen (PSC) preparation.

2.3. Fractionation of collagen types

PSC was extracted with 0.5 M acetic acid containing 11.0% (w/v) ammonium sulfate, as reported previously (Yata et al., 2001). After centrifugation at 10,000×*g* for 20 min, the supernatant was pooled. This procedure was repeated three times. After the final centrifugation, the resultant precipitate was collected for further purification and referred to as the P-fraction. On the other hand, the collagen in the supernatant (S-fraction) was salted out by adding ammonium sulfate to 20% (w/v). After centrifugation at 10,000×*g* for 20 min, the precipitate was pooled for further purification.

These fractions were further purified by cation-exchange column chromatography using SP-Toyopearl (M; Tosoh, Tokyo, Japan). Detailed elution conditions were described in figure captions. The effluent was monitored at 230 nm by a spectrophotometer (UV-9900; Tokyo Rikakikai Co., Tokyo, Japan). Appropriate fractions were pooled and dialyzed against distilled water, 20 mM disodium phosphate and distilled water, successively. Then they were freeze-dried for amino acid analysis and peptide mapping as described below.

2.4. Analytical methods

Nitrogen content of each tissue was determined by the micro-Kjeldahl method and was converted to crude protein using a factor of 6.25. For determination of collagen content, the muscle was extracted with NaOH solution as described above. The RS-AL was twice washed with distilled water and autoclaved for 1 h at 120 °C. An aliquot of the hot water-soluble fraction was hydrolyzed in 6 M HCl at 130 °C for 3 h. Hydroxyproline content in the hydrolysate was estimated according to the method of Woessner (1961). To convert the hydroxyproline content to collagen content the factors 10.7 and 10.3 were used for the ordinary and dark muscles, respectively. These factors had been calculated from the present results of amino acid analysis for type I collagens from the muscle tissues.

SDS-PAGE was performed according to the method of Laemmli (1970). The run was made at pH 8.8 in a 7.5% slab gel containing 0.1% SDS. Samples (about 2 μg) were applied to the gel and a molecular weight marker (SDS-6H; Sigma) was used as the standard.

For peptide mapping, the purified collagens (about 20 μg) were applied to the gel and digested with *Staphylococcus aureus* glutamyl endopeptidase (EC 3.4.21.19) or *Achromobactor lyticus* lysyl endopeptidase (EC 3.4.21.50) at an enzyme/substrate ratio of 1:50–1:100 (w/w) according to the method of Cleaveland, Fisher, Kirschner, and Laemmli (1977). Peptides generated by the protease digestion were separated by SDS-PAGE using a 12.5 or a 10.0% gel. The gel was stained for protein with Coomassie Brilliant Blue R-250, essentially according to the method of Fairbanks, Steck, and Wallah (1971).

For amino acid analysis, samples were hydrolyzed under vacuum with 6 M HCl at 150 °C for 1 h. Amino

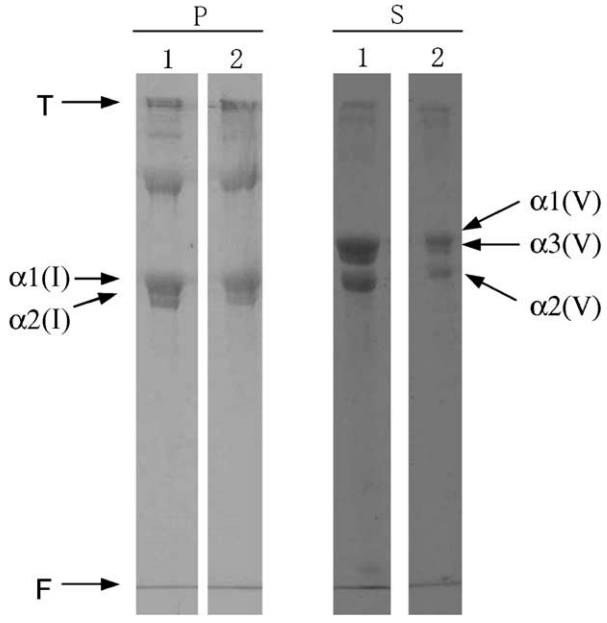

Fig. 1. SDS-PAGE patterns of P-fractions (P), and S-fraction (S) from the ordinary (1) and dark (2) muscles of Japanese amberjack. T = gel top and F = buffer front.

acid analysis was performed by the Pico-Tag system (Waters, Milford, MA, USA) according to the method of Sato et al. (1992).

3. Results and discussion

Collagen contents of ordinary and dark muscles of Japanese amberjack were 0.3 and 0.8% of wet tissue, and, 1.5 and 4.0% of crude protein, respectively. Fig. 1 shows SDS-PAGE patterns of P- and S-fractions from both tissues. The P-fractions from these tissues showed SDS-PAGE patterns typical of type I collagen, with two α chains, α 1(I) and α 2 (I). More than 90% of the total collagen was recovered in this fraction (major collagen). On the other hand, SDS-PAGE patterns of the S-fractions from these two tissues were similar to those of type V collagens, having three α chains designated as α1(V), α2(V), and α3(V), and less than 10% of the total collagen was recovered in this fraction (minor collagen).

P-fractions from both tissues were further purified by cation-exchange column chromatography using SP-Toyopearl. The chromatogram of P-fraction from ordinary muscle is shown in Fig. 2a. P-fraction was eluted in two peaks. These two elution peaks showed SDS-PAGE patterns typical of type I collagen having two α chains, α1(I) and α2(I). Similar elution patterns were obtained for the dark muscle (Fig. 2b). In both cases, little difference in peptide maps was detected between collagens of the two peaks (data not shown). In

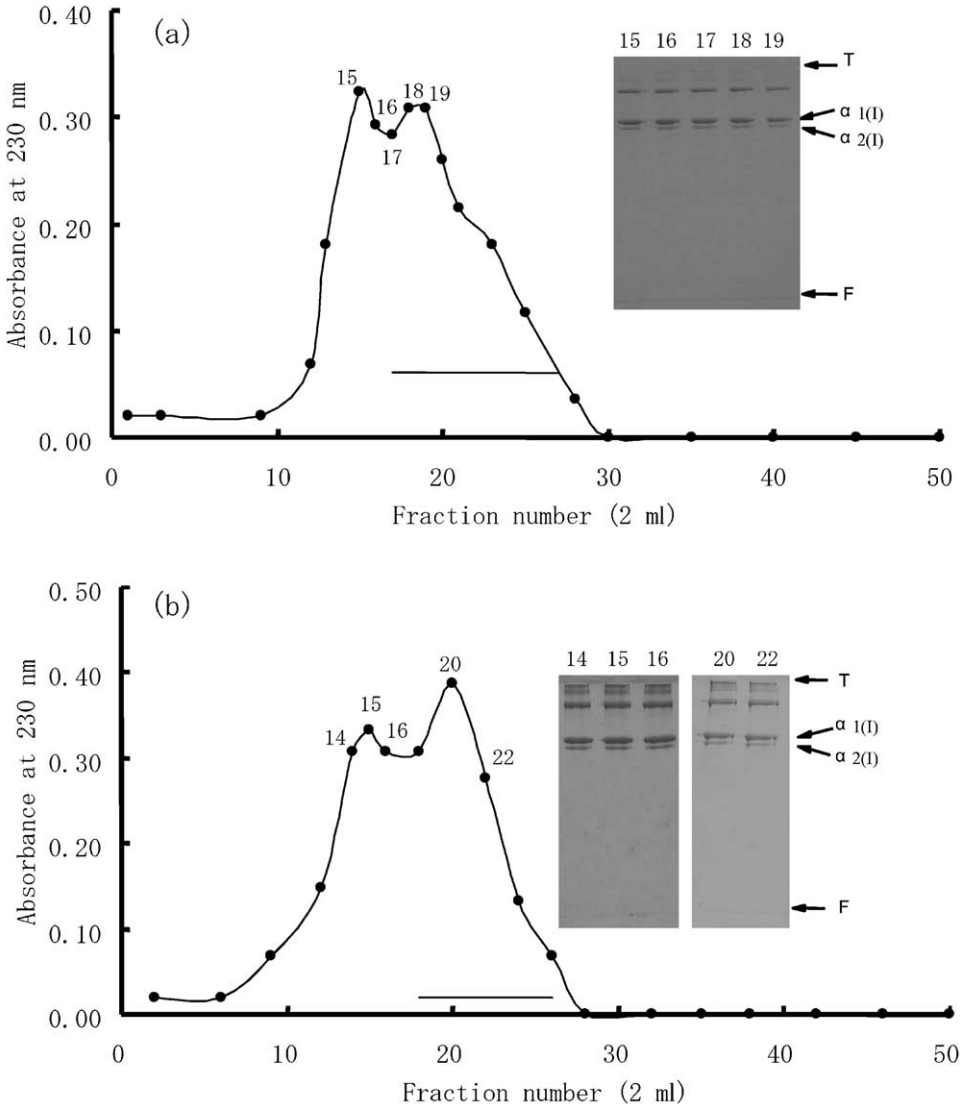

Fig. 2. Elution patterns of cation-exchange column chromatography of P fractions from the ordinary (a) and dark (b) muscles of Japanese amberjack, along with the SDS-PAGE patterns of the fractions indicated by fraction numbers. Underlined fractions were collected. P-fraction was dialyzed against 50 mM sodium acetate, pH 4.8, containing 2 M urea, and applied to the SP-Toyopearl column (1.5×5 cm) which had been equilibrated with the same buffer. Elution was achieved with a linear gradient from 0 to 1.0 M NaCl over a total volume of 100 ml at a flow rate of 1.0 ml/min. The effluent was monitored at 230 nm. Letters T and F are the same as in Fig. 1.

the present study, collagens in the main peaks were recovered for amino acid analysis.

As shown in Table 1, the amino acid composition of purified P-fractions from both muscles was similar to that of carp type I collagen (Sato et al., 1988), though there was somewhat of a difference between tissues in proline hydroxylation of type I collagen, as reported for skipjack by Zhu and Kimura (1991). Together with the results of precipitation properties by ammonium sulfate at acidic pH and SDS-PAGE, P-fractions might be classified as type I collagen. The peptide maps of the type I collagen were essentially similar between tissues (Fig. 4). In the present study, we showed at least two α components of type I collagens, which were separated by SDS-PAGE, from both muscles. We also observed minor differences in amino acid composition and peptide map of type I collagen between the tissues (Table 1, Fig. 4). Existence of two molecular forms, $[\alpha1(I)]_2\alpha2(I)$ and $\alpha1(I)\alpha2(I)\alpha3(I)$, were reported in fish type I collagen by Kimura et al. (1987). For skipjack, ordinary and dark muscle collagens were reported to exist as $[\alpha1(I)]_2\alpha2(I)$ (Zhu et al., 1991). However, Kimura et al. (1988) reported variation of subunit composition according to fish species. These results suggested that the relative proportion of the molecule $\alpha1(I)\alpha2(I)\alpha3(I)$ to the molecule $[\alpha1(I)]_2\alpha2(I)$ might be different between type I collagens from the ordinary and dark muscles.

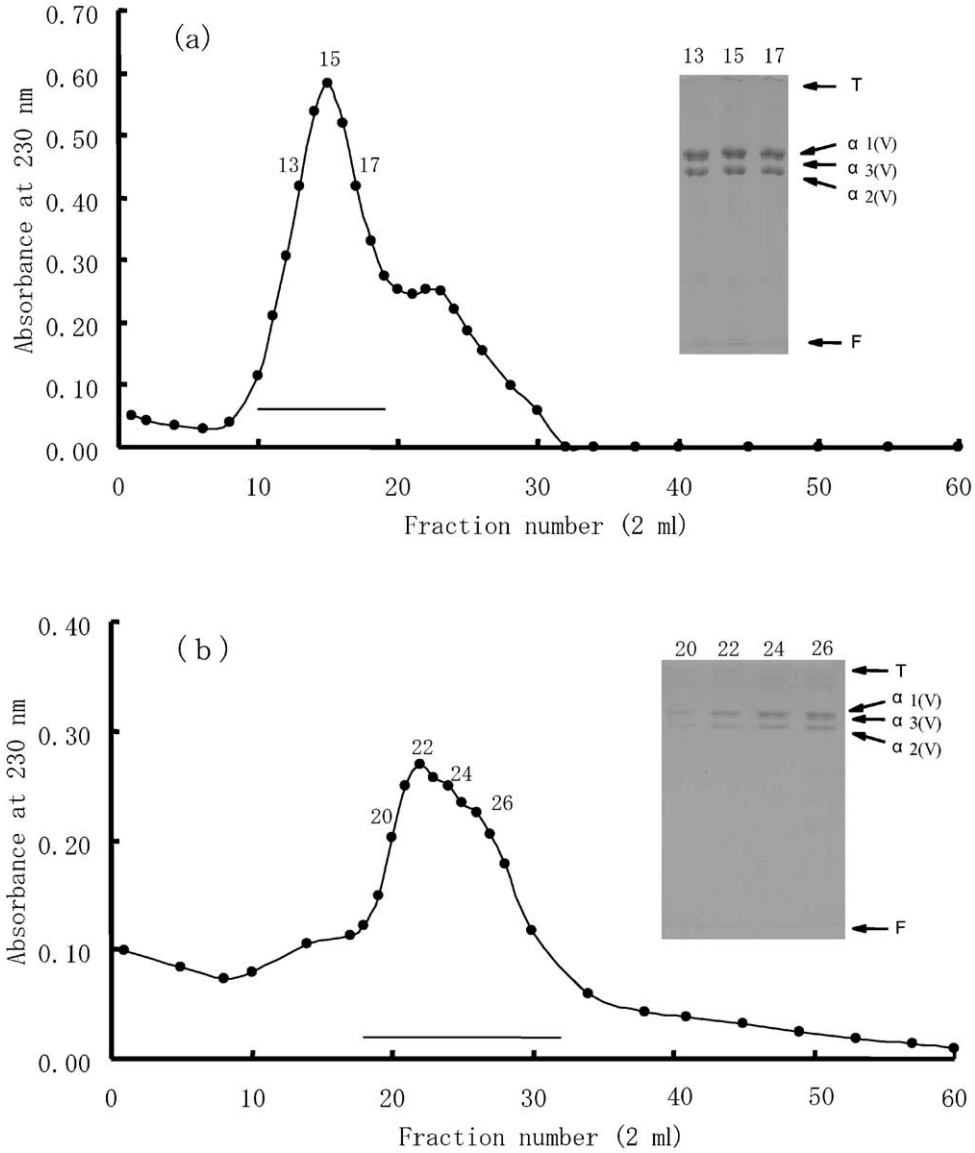

Fig. 3. Elution patterns of cation-exchange column chromatography of S- fractions from the ordinary (a) and dark (b) muscles of Japanese amberjack, along with the SDS-PAGE patterns of the fractions indicated by fraction numbers. S-fraction was dialyzed against 40 mM Tris–HCl, containing 2 M urea and 50 mM NaCl, pH 8.2 and applied to the SP-Toyopearl column (1.5×5 cm) which had been equilibrated with the same buffer. Elution was achieved with a linear gradient from 50 mM to 0.5 M NaCl over a total volume of 120 ml at a flow rate of 1.0 ml/min. The effluent was monitored at 230 nm. Letters T and F are the same as in Fig. 1.

Table 1
Amino acid composition of purified Type I and V collagens from the ordinary and dark muscles of Japanese amberjack (residues/1000 total residues)

	Type I collagen			Type V collagen		
	Japanese amberjack[a]		Carp[b]	Japanese amberjack[a]		Carp[b]
	Ordinary muscle	Dark muscle	Ordinary muscle	Ordinary muscle	Dark muscle	Ordinary muscle
Asp	43.4±2.2	44.0±0.9	41	46.4±0.8	44.2±1.0	41
Glu	75.2±2.7	75.9±0.4	72	94.3±1.0	95.2±2.2	93
Hyp	68.2±0.8	71.3±0.7	85	84.8±0.7	80.3±0.7	87
Ser	41.5±0.7	39.2±0.5	37	27.9±0.6	25.7±0.4	41
Gly	356.2±2.9	356.4±1.1	339	354.2±2.1	341.3±3.2	326
His	4.5±0.1	4.4±0.1	5	8.9±0.1	8.1±0.1	10
Arg	49.3±0.3	50.9±1.7	52	41.2±0.3	39.8±0.1	48
Thr	35.5±0.3	34.8±0.1	26	28.5±0.2	22.0±0.2	32
Ala	122.8±0.7	122.9±0.9	121	57.7±0.3	62.3±0.8	62
Pro	104.9±1.9	103.1±2.1	112	116.9±3.9	118.9±0.4	110
Tyr	2.0±0.2	1.8±0.1	3	2.9±0.1	2.3±0.2	6
Val	19.6±0.1	19.7±0.3	16	21.4±0.9	21.4±0.3	19
Met	3.4±0.6	1.8±0.1	12	11.0±0.1	8.7±0.2	2
Ile	10.6±0.3	10.6±0.4	10	14.5±0.2	16.4±0.2	20
Leu	19.5±0.9	19.1±0.5	22	37.5±0.2	43.1±0.1	34
Hyl	5.1±0.3	5.1±0.1	7	24.8±1.9	36.3±1.2	30
Phe	11.4±0.4	11.8±0.4	13	9.7±0.2	12.2±0.1	14
Lys	26.7±0.4	27.2±1.7	26	17.5±2.8	22.1±1.1	26

[a] The average±S.D. of three determinations for the same sample preparations.
[b] Sato et al. (1988).

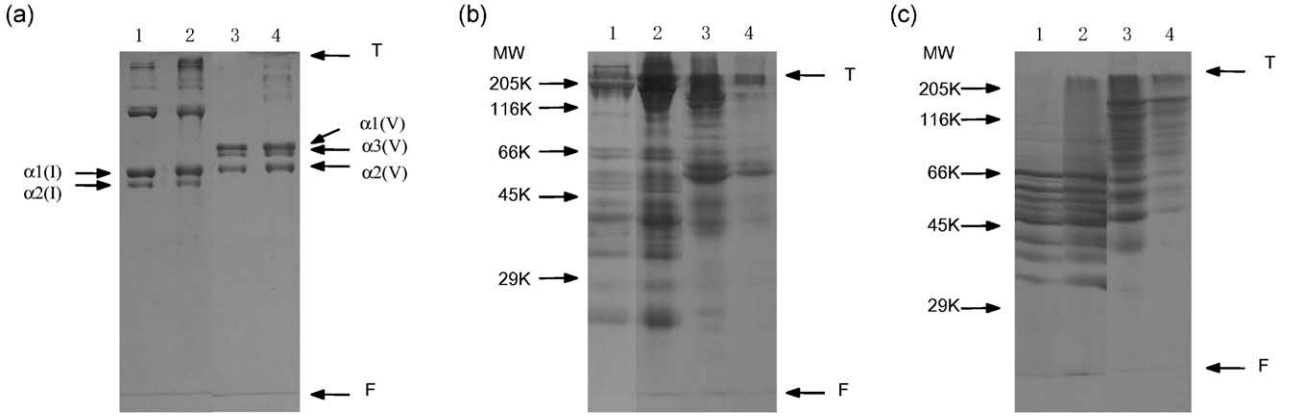

Fig. 4. SDS-PAGE patterns (a) of the purified type I (1 and 2) and type V (3 and 4) collagens from the ordinary (1 and 3) and dark (2 and 4) muscles of Japanese amberjack, and their peptide maps of glutamyl (b, 12.5% gel) and lysyl (c, 10% gel) endopeptidase digests. Arrows (MW 205, 116, 66, 45, and 29 kDa) show the molecular weights and positions of the standard proteins. Letters T and F are the same as in Fig. 1.

The S-fractions from the ordinary and dark muscles of Japanese amberjack were further purified by cation-exchange chromatography (Fig. 3). The main fraction in each chromatogram showed a SDS-PAGE pattern typical of type V collagen. However, elution position was different between the tissues.

As shown in Table 1, purified S-fractions from both tissues showed typical features of type V collagen in amino acid composition, having relatively lower content of alanine and higher content of hydroxylysine than those of type I collagen (Sato et al., 1988). From the data on electrophoretic patterns, precipitation properties by ammonium sulfate at acidic pH, and amino acid composition, S-fractions in both tissues may be classified as type V collagen, though the contents of some amino acids such as threonine, methionine, and hydroxylysine differed among tissues. The peptide maps of the type V collagens were essentially similar between tissues (Fig. 4). Major molecular forms of type V collagen in eel *Anguila japonica* muscle was demonstrated to be $[\alpha1(V)]_2\alpha2(V)$ and $\alpha1(V)\alpha3(V)\alpha4(V)$ by Sato, Sakuma, Ohtsuki, and Kawabata (1994). They also suggested the presence of α4(V) chain in other fish, which can not be distinguished from the α2(V) chain on SDS-PAGE. The

differences in the composition of molecular forms of type V collagen in the present study might cause differences in the elution position and amino acid composition between tissues.

In the present study, we classified the major and minor collagens in ordinary and dark muscles in Japanese amberjack as type I and V collagens. These collagens might relate to textural changes of each muscle during chilled storage and subsequent processing, such as heating. A study on the relationship between collagen and textural changes of each muscle during heating is now in progress.

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