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Identification and characterization of molecular species of collagen in ordinary muscle and skin of the Japanese flounder *Paralichthys olivaceus*

Maki Nishimoto, Ryoko Sakamoto, Shoshi Mizuta *, Reiji Yoshinaka

Department of Marine Bioscience, Faculty of Biotechnology, Fukui Prefectural University, Obama, Fukui 917-0003, Japan

Abstract

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

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1. Introduction

Collagen, as the major constituent of fish connective tissue, has been demonstrated to exist in different genetic forms (Kimura, 1997). In ordinary muscle and skin of fish, at least 2 types of collagen, type I and type V collagens, were found to be major and minor collagens, respectively (Kimura, Zhu, Matsui, Shijoh, & Takamizawa, 1988; Sato, Yoshinaka, Sato, Itoh, & Shimizu, 1988; Sato, Yoshinaka, Itoh, & Sato, 1989; Yata, Yoshida, Fujisawa, Mizuta, & Yoshinaka, 2001).

Collagen plays an important role in the development of the physical properties of fish meat. The contribution of collagen to the texture of raw fish meat has been reported: the higher the collagen content was, the firmer the meat was (Hatae, Tobimatsu, Takeyama, & Matsumoto, 1986; Sato, Yoshinaka, Sato, & Shimizu, 1986). The effect of collagen on fish muscle tenderization during *post-mortem* aging has been studied (Ando, Toyohara, Shimizu, & Sakaguchi, 1993). Recently, enzymatic degradation of type V collagen was found to be responsible for its tenderization (Sato et al., 1997). In addition, collagen is denatured and converted into gelatin during cooking. Gelatin has the peculiar characteristic of producing thermoreversible gels at chilled temperatures. Fish broth, generated after cooking, forms a gel, called "Nikogori" (fish aspic), by cooling and is sometimes eaten preferably in Japan. As cooked fish flesh is often eaten with skin, from which Nikogori is mainly made, it is also necessary to study the properties of fish skin collagen.

Although much work has been done on collagens in different fish species, there is little information of the collagen types in heterosomata, except for a report on type I collagen from Northern dab *Limanda herzensteini* (Kimura, Ohno, Miyauchi, & Uchida, 1987). In this paper, we tried to classify the collagen types in ordinary muscle and skin of the Japanese flounder (*Paralichthys olivaceus*), which is a popular fish and which is cultured widely in Japan, to clarify food functions of their collagens.

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* Corresponding author. Tel.: +81-770-52-6300; fax: +81-770-52-6003.

E-mail address: mizuta@fpu.ac.jp (S. Mizuta).

2. Materials and methods

2.1. Materials

Japanese flounder *P. olivaceus* (average body weight 292 g), obtained alive from the Research Center for Marine Bioresources, Faculty of Biotechnology of Fukui Prefectural University (Fukui, Japan), was gutted and used immediately for the experiment. For analyses of proximate composition, specimens of the Japanese flounder (average body weight 720 g) obtained from the Fukui Prefectural Sea Farming Center (Fukui, Japan) were gutted, skinned, filleted and stored at −80 °C prior to the experiment. All other reagents were of analytical grade.

2.2. Preparation of collagens

All procedures were performed in a cold room at 5 °C. The fish were skinned and filleted. Muscle tissue (about 900 g) was homogenized with 2 vol. (v/w) of 0.1 M NaOH, using a non-bubbling homogenizer (NS-2; Nissei, Tokyo, Japan). The suspension was stirred overnight and centrifuged at 10,000g for 20 min. To the residue, 10 vol. of 0.1 M NaOH were added and the suspension was stirred overnight. This procedure was repeated 3 or 4 times.

The skin (about 200 g) was scaled, cut into small pieces with scissors, and homogenized with 10 vol. (v/w) of 0.1 M NaOH in a POLYTRON homogenizer (Kinematica, Luzern, Switzerland). The suspension was stirred overnight and centrifuged at 10,000g for 20 min. The resultant precipitate was rehomogenized with 5 vol. of 0.1 M NaOH and stirred overnight. This procedure was repeated 3 or 4 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 from the ordinary muscle was digested with pepsin at an enzyme/substrate ratio of 1/50 (w/w) for 24 h at 5 °C. In the case of skin, the RS-AL was digested with pepsin at an enzyme/substrate ratio of 1/1000 (w/w) for 24 h at 5 °C. Both pepsin digests were centrifuged at 10,000g for 20 min. 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 10,000g 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 10,000g for 20 min, the supernatant was pooled. This procedure was repeated 3 times. After the final centrifugation, the re-sultant precipitate was collected for further purification and is referred to as the P-fraction. On the other hand, the collagen in the supernatant was salted out by adding ammonium sulfate to 20% (w/v). After centrifugation at 10,000g for 20 min, the precipitate was pooled for further purification as the S-fraction.

These fractions were further purified by cation-exchange column chromatography, using phosphocellulose P-11 (Whatman, Kent, England). Detailed elution conditions are described in figure captions (see below). The effluent was monitored at 230 nm by a spectrophotometer (UV-9900; Tokyo Rikakikai Co., Tokyo, Japan). Appropriate fractions were pooled, salted-out by 2.0 M NaCl in 0.5 M acetic acid, 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

The nitrogen content of each tissue was determined by the micro-Kjeldahl method and was converted to crude protein content using a factor of 6.25. For determination of collagen content, the muscle was extracted with NaOH solution twice as described above. The RS-AL was washed with distilled water twice and auto-claved for 1 h at 120 °C. The skin was 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 determined according to the method of Woessner (1961). To convert the hydroxyproline content to collagen content, the factor 10.2 was used for the ordinary muscle and skin. This factor had been calculated from the present results
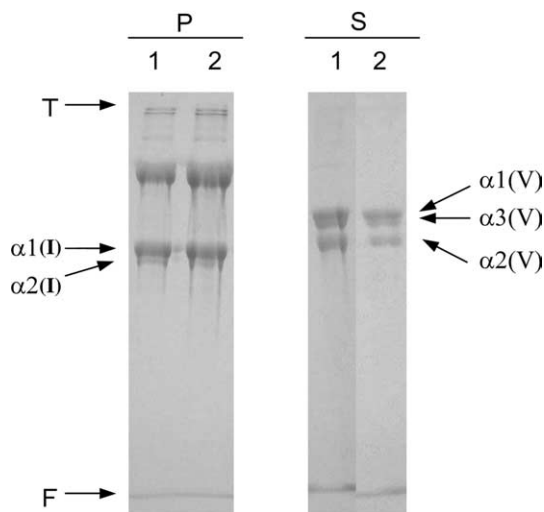

Fig. 1. SDS–PAGE patterns of P-fractions (P), and S-fraction (S) from the ordinary muscle (1) and skin (2) of Japanese flounder. T = gel top and F = buffer front.

of amino acid analysis for type I collagens from the ordinary muscle and skin.

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 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 *Achromobacter 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 Cleveland, Fischer, 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 ac-

cording to the method of Fairbanks, Steck, and Wallach (1971).

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

The collagen contents of ordinary muscle and skin of Japanese flounder were 1.1% and 25.7% of wet tissue and 4.6% and 77.6% 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,
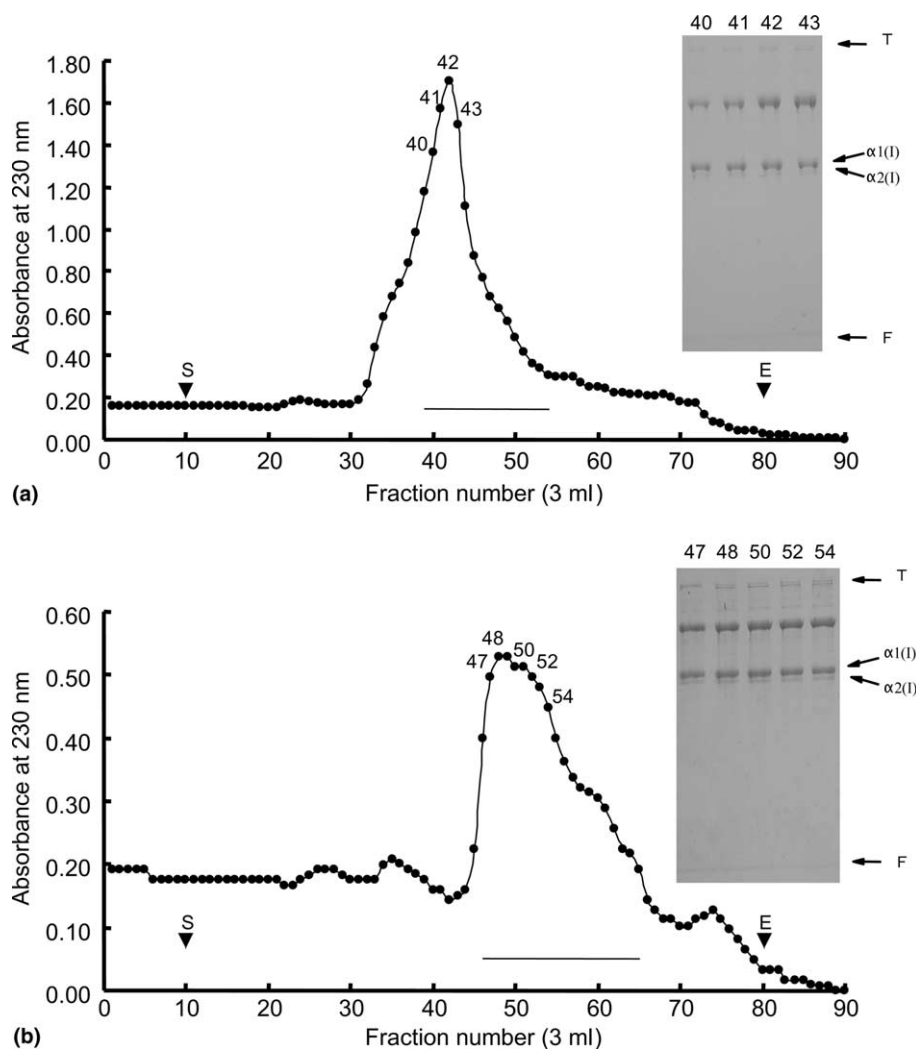

Fig. 2. Elution patterns of cation-exchange column chromatography of P-fractions from the ordinary muscle (a) and skin (b) of Japanese flounder, along with the SDS–PAGE patterns of the fractions indicated by fraction numbers. P-fraction was dialyzed against 50 mM sodium acetate, pH 4.8, containing 2 M urea and applied to phosphocellulose P-11 column (2.5 × 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 210 ml at a flow rate of 1.0 ml/min. The effluent was monitored at 230 nm. Underlined fractions were collected. S = gradient start and E = gradient end. Letters T and F are the same as in Fig. 1.

showing two α chains, α1(I) and α2(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).

The P-fractions from both ordinary muscle and skin were further purified by cation-exchange column chromatography, using phosphocellulose P-11. A chromatogram of the P-fraction from the ordinary muscle is shown in Fig. 2(a). The P-fraction was eluted as a single peak with a shoulder. This peak 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 skin (Fig. 2(b)). In the present study, collagens in the main peaks were recovered for further analysis.

As shown in Table 1, the amino acid compositions of purified P-fractions from both tissues were similar to that of carp type I collagen (Sato et al., 1988). Together with the results of precipitation properties by ammonium sulfate at acidic pH and SDS–PAGE, P-fractions might be classified type I collagen. The peptide maps of the type I collagens 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 tissues. We also observed the minor differences in amino acid composition of type I collagen between both tissues (Table 1). The existence of two molecular forms, $[\alpha1(I)]_2\alpha2(I)$ and α1(I)α2(I)α3(I), was reported in fish type I collagen by

Kimura et al. (1987). These results suggested that the relative proportion of the molecule α1(I)α2(I)α3(I) to the molecule $[\alpha1(I)]_2\alpha2(I)$ might be different between type I collagens from the ordinary muscle and skin.

The S-fractions from the ordinary muscle and skin of Japanese flounder were further purified by cation-exchange chromatography (Fig. 3). The S-fraction of the ordinary muscle was eluted in 2 peaks (Fig. 3(a)). The SDS–PAGE pattern of the second peak showed that of type V collagen, while that of the first peak did not show any protein band. The second peak was collected and used for further analysis. The elution pattern of the S-fraction from the skin was similar to that of the ordinary muscle (Fig. 3(b)). The main elution peak showed a SDS–PAGE pattern, typical of type V collagen, having three α chains designated as α1(V), α2(V) and α3(V).

As shown in Table 1, the purified S-fractions from both tissues showed typical features of type V collagen in amino acid composition, having relatively lower contents of alanine and higher contents 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 differed between 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 were demonstrated by Sato, Sakuma, Ohtsuki, and Kawabata (1994), to be $[\alpha1(V)]_2\alpha2(V)$ and α1(V)α3(V)α4(V). They also suggested the presence of α4(V) chain in other

Table 1
Amino acid composition of purified type I and type V collagens from the ordinary muscle and skin of Japanese flounder (residues/1000 total residues)

	Type I collagen			Type V collagen		
	Japanese flounder[a]		Carp[b]	Japanese flounder[a]		Carp[b]
	Ordinary muscle	Skin	Ordinary muscle	Ordinary muscle	Skin	Ordinary muscle
Asp	44.4 ± 1.2	42.9 ± 0.4	41	45.7 ± 0.6	46.6 ± 1.0	41
Glu	73.2 ± 0.3	73.5 ± 0.5	72	89.2 ± 0.7	96.4 ± 2.9	93
Hyp	72.5 ± 0.3	71.8 ± 0.3	85	77.7 ± 1.3	77.9 ± 2.3	87
Ser	35.7 ± 0.8	39.0 ± 0.5	37	33.4 ± 0.3	38.3 ± 0.8	41
Gly	360.4 ± 1.5	363.8 ± 1.8	339	337.2 ± 2.5	339.2 ± 2.1	326
His	3.1 ± 0.1	3.1 ± 0.1	5	8.8 ± 0.2	10.6 ± 0.4	10
Arg	51.4 ± 0.0	51.0 ± 1.0	52	45.6 ± 1.3	40.9 ± 1.3	48
Thr	24.2 ± 0.5	25.2 ± 0.6	26	33.7 ± 0.3	33.7 ± 0.4	32
Ala	126.8 ± 1.3	126.9 ± 1.8	121	60.0 ± 0.4	54.1 ± 1.1	62
Pro	108.3 ± 0.9	107.5 ± 1.3	112	112.0 ± 1.1	107.0 ± 3.8	110
Tyr	1.8 ± 0.3	1.7 ± 0.1	3	2.8 ± 0.1	2.5 ± 0.2	6
Val	18.2 ± 0.3	18.6 ± 0.2	16	19.8 ± 0.3	20.7 ± 1.0	19
Met	14.1 ± 0.0	13.4 ± 0.3	12	5.9 ± 1.3	9.2 ± 0.6	2
Ile	7.4 ± 0.4	7.5 ± 0.2	10	13.6 ± 0.1	13.3 ± 1.2	20
Leu	18.7 ± 0.2	17.0 ± 0.2	22	35.0 ± 0.9	34.6 ± 2.3	34
Hyl	7.9 ± 0.2	6.3 ± 0.1	7	37.6 ± 2.3	43.7 ± 0.1	30
Phe	12.9 ± 0.3	10.8 ± 0.1	13	11.1 ± 0.3	10.4 ± 1.1	14
Lys	19.2 ± 0.7	19.9 ± 0.4	26	31.1 ± 0.4	20.9 ± 1.6	26

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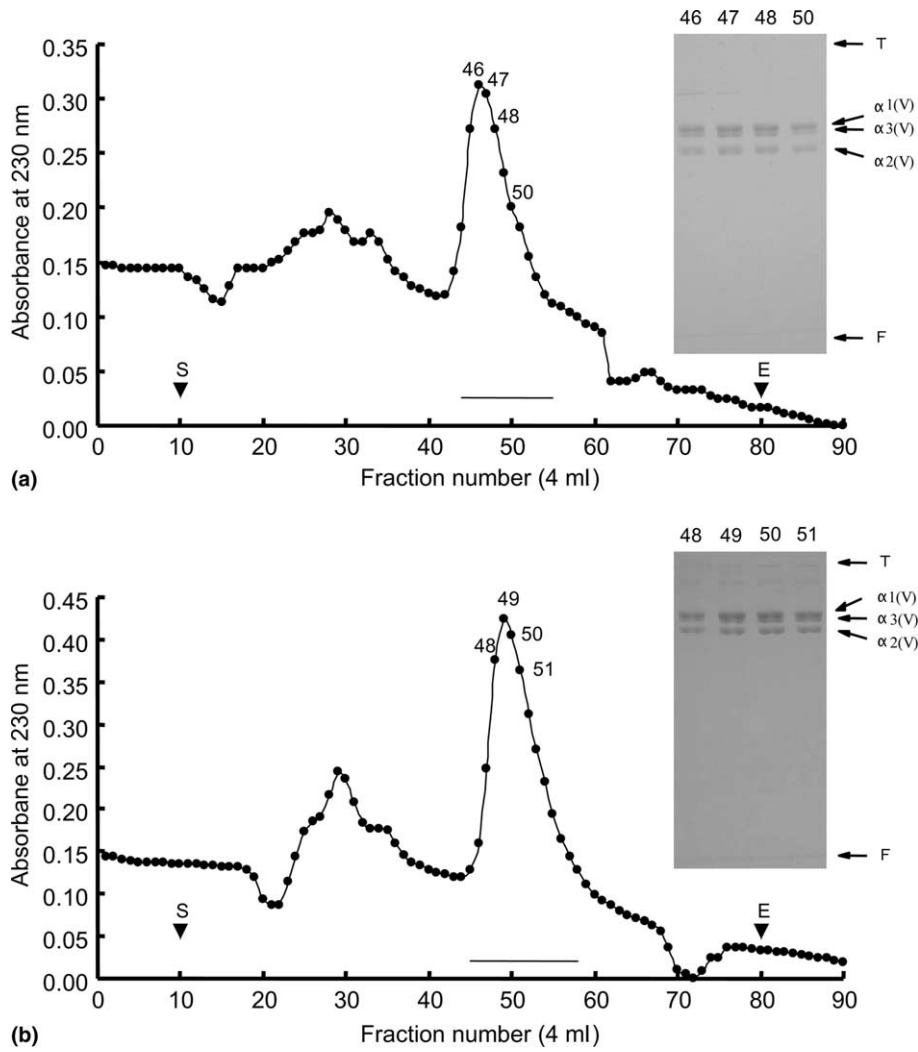[b] Sato et al. (1988).

Fig. 3. Elution patterns of cation-exchange column chromatography of S-fractions from the ordinary muscle (a) and skin (b) of Japanese flounder, along with the SDS–PAGE patterns of the fractions indicated by fraction numbers. S-fraction was dialyzed against 50 mM sodium phosphate, pH 6.8, containing 2 M urea and applied to phosphocellulose P-11 column (2.5 × 5 cm) which had been equilibrated with the same buffer. Elution was achieved with a linear gradient from 0 to 0.8 M NaCl over a total volume of 300 ml at a flow rate of 1.0 ml/min. The effluent was monitored at 230 nm. Underlined fractions were collected. Letters T, F, S and E are the same as in Fig. 2.
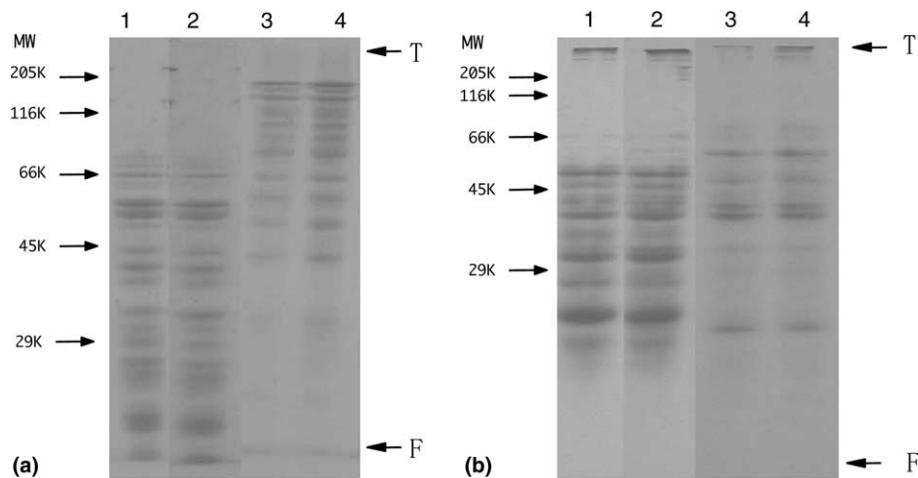

Fig. 4. Peptide maps of lysyl (a, 10% gel) and glutamyl (b, 12.5% gel) endopeptidase digests of purified type I (1 and 2) and type V (3 and 4) collagens from the ordinary muscle (1 and 3) and skin (2 and 4) of Japanese flounder. Arrows (205, 66, 45 and 29 kDa) show the molecular weights and positions of the standard proteins. Letters T and F are the same as in Fig. 1.

fishes, which cannot be distinguished from α2(V) chain on SDS–PAGE. The differences in the composition of molecular forms of type V collagen in the present study might cause the differences in amino acid composition between tissues.

In the present study, we classified types I and V collagens in ordinary muscle and in skin of the Japanese flounder and clarified their chemical compositions, such as the amino acids. Further studies must be carried out to demonstrate how these collagens relate to food processing parameters. To solve these problems, studies on changes of these collagens during heat treatment are now in progress.

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