Identification and Characterization of Molecular Species of Collagen in Fish Skin

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ABSTRACT: Pepsin-solubilized collagen (PSC) prepared from the skin of 3 fish species—common horse mackerel, yellow sea bream, and tiger puffer—were separated into 2 fractions, major and minor, by ammonium sulfate precipitation. These collagen fractions were further purified by phosphocellulose column chromatography. From the results of SDS-PAGE, peptide mapping, and amino-acid analysis, the purified major and minor collagens were identified to be type I and V collagens, respectively. These results suggest that type V collagen might be widely present in fish skin as a minor collagen.

Key Words: collagen, fish, skin, amino-acid analysis, type V collagen

Introduction

IN POSTHARVEST DISTRIBUTION AND PROCESSING, THE SKIN tissue of commercial fish plays important functions in maintaining the form of the fish body and avoiding the degradation of muscle structure. For some fish species, skin is also important as one of the edible parts. Small-size cooked fish is often eaten with the skin. In Japan, the boiled skin of fish such as tiger puffer (*Takifugu rubripes*) is eaten to enjoy its texture. Skin, in general, consists mainly of 2 layers, the epidermis and dermis (Lagler and others 1977). The dermis is a thick connective tissue with a considerable amount of collagen fibers.

In intramuscular connective tissue of fish, at least, type I and V collagens have been identified as major and minor collagens, respectively (Kimura and others 1988; Sato and others 1988, 1989). Enzymatic degradation of type V collagen was reported to be responsible for the postharvest softening of fish muscle such as rainbow trout (*Oncorhychus mykiss*) and sardine (*Sardinops melanosticta*) (Sato and others 1991, 1997). On the other hand, collagen attention in fish skin has been almost solely restricted to type I collagen (Kimura and others 1987). The presence of minor collagen types is still unknown. Thus, the purpose of our study was to identify collagen types in the skin of fish. For comparison, major and minor collagens from the ordinary muscle of the same fish species were also purified and identified.

Materials and Methods

Materials

Fresh individuals of common horse mackerel (*Trachurus japonicus*) and yellow sea bream (*Dentex tumifroms*) (average body weight 96 and 97 g, respectively) captured by trawl net were obtained from a local market and used immediately. Tiger puffers (average body weight 186 g), reared at the Aquaculture Research Station of Fukui Prefectural Univ., were gutted and stored at −40 °C until needed. Pepsin (crystallized and lyophilized, EC 3.4.23.1) was obtained from Sigma Chemical Co. (St.Louis, Mo., U.S.A.). All other reagents were of analytical grade.

Preparation of Collagens

All procedures were performed in a cold room at 5 °C. The fish were skinned and filleted. The skin was scaled, cut into small pieces with scissors, and homogenized with 10 volumes of 0.1 M NaOH in a POLYTRON homogenizer (Kinematica, Luzern, Switzerland). This extraction was done to remove noncollagenous proteins and to prevent the effect of endogenous proteases on collagen (Sato and others 1987). The suspension was stirred overnight and centrifuged at 10,000 × g for 20 min. The resultant precipitate was rehomogenized with 20 volumes of 0.1 M NaOH and stirred overnight. This procedure was repeated 3 or 4 times. Muscle tissue (about 300 g) was homogenized with 3 volumes of 0.1 M NaOH using a nonbubbling homogenizer (NS-2; Nissei, Tokyo, Japan). The suspension was stirred overnight and centrifuged at 10,000 × g for 20 min. To the residue, 10 volumes of 0.1 M NaOH was added, and the suspension was 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. In the case of skin, the suspension was stirred with pepsin, which cleaved the nonhelical region, telopeptide, at an enzyme/substrate ratio of 1/1000 (w/w) for 24 h at 5 °C. The RS-AL from the ordinary muscle was digested with pepsin at an enzyme/substrate ratio of 1/50-1/20 (w/w) for 24 h at 5 °C. Both were centrifuged at 10,000 × g 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 10,000 × g for 20 min, the resultant precipitate was used as a pepsin-solubilized collagen (PSC) preparation.

Fractionation of Collagens into Types

PSC was fractionated according to the method of Sato and others (1991) with a slight modification. PSC was extracted with 0.5 M acetic acid containing 11.0 % (w/v) ammonium sulfate, except in the case of yellow sea bream, where a concentration of 10.5% (w/v) ammonium sulfate was adopted for the fractionation because sufficient recovery of minor collagens was not achieved with 11.0% (w/v) ammonium sulfate. After centrifugation at 10,000 × g for 20 min, the supernatant was pooled (S-fraction). To the precipitate, 0.5 M acetic acid containing 10.5 % or 11.0 % (w/v) ammonium sulfate was added, and the suspension was stirred overnight. This procedure was repeated 3 times. After centrifugation at

Table 1—Amino-acid composition of purified major collagens from skin and ordinary muscle of common horse mackerel, yellow sea bream and tiger puffer (residues / 1000 total residues)

	Common horse mackerel		Yellow sea bream		Tiger puffer		Carp type I*[1]
	skin	ordinay muscle	skin	ordinary muscle	skin	ordinary muscle	muscle
Asp	42.9±1.4	42.4±0.2	40.7±0.9	36.1±0.7	44.6±0.4	46.9±0.1	41
Glu	72.5±0.6	70.1±0.4	72.6±1.2	67.2±4.8	68.0±0.3	68.9±0.4	72
Hyp	70.7±0.4	73.0±0.2	75.5±1.1	79.1±1.2	73.1±0.2	75.2±0.9	85
Ser	35.8±0.2	32.7±1.0	41.2±0.6	40.6±0.3	42.9±0.4	41.4±0.3	37
Gly	361.5±0.3	362.5±1.3	351.3±2.0	350.7±0.6	349.9±2.1	346.9±1.6	339
His	4.4±0.0	3.9±0.0	3.8±0.2	3.1±0.1	3.6±0.1	3.5±0.0	5
Arg	53.0±0.4	52.0±0.5	52.0±1.3	50.7±0.7	52.7±0.1	51.1±0.9	52
Thr	30.1±0.4	29.1±0.1	29.8±0.5	31.0±0.1	29.1±0.5	29.1±0.7	26
Ala	120.6±0.7	127.0±1.3	124.7±1.2	125.4±1.4	117.5±1.6	118.2±1.2	121
Pro	115.2±2.2	110.9±0.2	110.7±0.4	114.0±2.1	112.5±0.3	108.8±0.4	112
Tyr	1.5±0.1	1.8±0.1	2.0±0.0	2.2±0.2	1.6±0.1	2.0±0.0	3
Val	16.1±0.4	15.0±0.2	16.3±0.3	19.2±0.7	21.7±0.0	22.3±0.1	16
Met	10.1±0.9	10.2±0.2	11.4±2.0	17.3±0.1	14.0±0.2	13.0±0.5	12
Ile	8.2±0.2	8.7±0.1	6.9±0.1	8.3±0.9	7.4±0.0	8.2±0.1	10
Leu	18.7±0.1	19.8±0.3	17.0±0.7	16.9±1.5	15.4±0.2	16.8±0.2	22
Hyl	7.5±0.1	5.7±0.3	6.2±0.7	6.7±0.7	4.8±0.2	8.6±0.1	7
Phe	11.2±0.0	11.5±0.1	12.2±0.5	11.1±1.4	12.3±0.1	13.1±0.2	13
Lys	23.7±0.9	23.7±1.1	25.6±1.3	20.3±3.2	29.0±1.8	25.9±0.1	26

*[1] Sato and others 1988.
The average ± S.D. of three determinations for the same sample preparations.

Table 2—Amino-acid composition of purified minor collagens from skin and ordinary muscle of common horse mackerel, yellow sea bream and tiger puffer (residues / 1000 total residues)

	Common horse mackerel		Yellow sea bream		Tiger puffer		Carp type V*[1]
	skin	ordinary muscle	skin	ordinary muscle	skin	ordinary muscle	muscle
Asp	49.8±0.6	45.5±0.9	43.5±3.4	43.7±1.2	48.5±0.7	48.0±1.3	41
Glu	95.4±0.3	93.1±0.6	95.3±0.2	84.1±4.6	82.8±0.8	91.7±1.2	93
Hyp	78.0±0.6	75.9±0.6	84.9±0.3	86.0±0.8	85.7±0.0	82.8±0.5	87
Ser	33.5±0.3	31.6±0.3	34.6±0.2	34.9±1.4	38.2±0.3	35.8±0.4	41
Gly	354.7±2.5	353.6±0.5	347.4±2.1	346.8±4.3	342.1±1.2	337.1±1.8	326
His	11.2±0.1	10.1±0.2	8.5±0.1	8.2±0.3	7.6±0.1	7.4±0.1	10
Arg	43.9±0.2	41.9±0.8	42.3±0.2	42.4±0.5	50.6±0.2	43.3±0.4	48
Thr	26.3±0.1	25.9±0.4	29.6±0.2	30.3±0.3	32.7±0.4	32.4±0.5	32
Ala	51.1±1.0	55.4±0.3	52.9±0.1	51.8±0.7	64.5±0.9	52.7±0.6	62
Pro	118.2±0.5	115.8±1.4	115.0±1.3	117.3±2.3	119.2±1.2	113.2±1.0	110
Tyr	3.1±0.1	2.4±0.1	2.4±0.1	3.2±0.4	3.8±0.2	2.5±0.2	6
Val	23.6±0.6	20.8±0.4	21.3±0.2	27.7±1.2	16.7±0.1	19.2±0.0	19
Met	7.1±0.8	7.5±0.1	9.4±0.5	17.7±2.5	10.7±0.0	8.7±0.0	2
Ile	14.4±0.3	13.0±0.2	14.5±0.2	15.7±1.2	11.9±0.1	15.4±0.0	20
Leu	38.3±0.9	36.0±0.2	37.6±0.3	36.5±1.4	29.6±0.3	38.1±0.5	34
Hyl	23.6±0.7	44.2±0.5	33.3±0.4	28.8±1.8	24.7±0.2	44.1±0.7	30
Phe	11.2±0.3	10.7±0.1	11.0±0.1	10.6±0.2	11.0±0.1	11.0±0.1	14
Lys	16.7±1.1	16.5±0.9	16.8±0.1	14.2±0.3	19.6±0.3	16.9±0.3	26

*[1] Sato and others 1988.
The average ± S.D. of three determinations for the same sample preparations.

10,000 × g for 20 min, the resultant precipitate was collected and referred to as the P-fraction.

P-fraction was dissolved in and dialyzed against 50 mM sodium acetate, pH 4.8, containing 0.2 M NaCl and 2 M urea, and applied to a column of phosphocellulose P-11 (Whatman, Kent, England) that had been equilibrated with the same buffer. Elution was achieved with a linear gradient from 0.2 to 1.0 M NaCl over a total volume of 240 mL at a flow rate of 1.0 mL/min.

To S-fraction, ammonium sulfate was added to a final concentration of 20 % (w/v). The suspension was stirred, left standing overnight, and centrifuged at 10,000 × g for 20 min. The resultant precipitate was dissolved in and dialyzed against 50 mM sodium phosphate, pH 6.8, containing 2 M urea, and applied to a column of phosphocellulose P-11 that 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 360 mL at a flow rate of 1.0 mL/min.

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 and 20 mM disodium phosphate. Then they were freeze-dried for amino-acid analysis and peptide mapping.

SDS-PAGE and Peptide Mapping

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 (4-50 μg/well) were applied to the gel, and molecular weight marker (SDS-6H; Sigma) was used as the standard.

The purified collagens (15-20 μg/well) were applied to the gel and digested with *Staphylococcus aureus* V-8 protease (EC 3.4.21.19) or *Achromobactor lyticus* lysyl endopeptidase (EC 3.4.21.50) at an enzyme/substrate ratio of 1:40-1:100 (w/w) according to the method of Cleaveland and others (1977). Peptides generated by the protease digestion were separated by SDS-PAGE using a 12.5% or a 10% gel.

The gel was stained for protein with Coomassie brilliant blue R-250 essentially according to the method of Fairbanks and others (1971).

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, Mass., U.S.A.) according to the method of Sato and others (1992).

Results and Discussion

FIGURE 1 SHOWS SDS-PAGE PATTERNS OF RS-AL, PSC, P-fraction, and S-fraction from the skin and the ordinary muscle of common horse mackerel, along with type I collagen from the carp (*Cyprinus carpio*) ordinary muscle. In RS-AL and PSC, 2 α bands corresponding to α1(I) and α2(I) components were observed as well as faint bands at the position around the asterisk (*). SDS-PAGE patterns of P-fractions from the skin and the ordinary muscle were similar to that of type I collagen from the carp ordinary muscle. More than 90% of the total collagen was collected in this fraction and referred to as a major collagen. On the other hand, the SDS-PAGE patterns of S-fractions from both tissues of common horse mackerel (Fig. 1, lanes S) were quite similar to that of type V collagen isolated from the carp ordinary muscle, which had been reported by Sato and others (1988). The minor α chain-sized components observed in the RS-AL and PSC preparations seemed to be concentrated in this fraction. Less than 10% of the total collagen was collected in this fraction and referred to as a minor collagen.

The major collagen from the skin of common horse mackerel was further purified by phosphocellulose column chromatography (Fig. 2a). The 1st large peak showed the typical SDS-PAGE pattern of type I collagen with 2 α bands, α1(I) and α2(I). Existence of 2 forms, $[\alpha1(I)]_2$ α2(I) and α1(I) α2(I) α3(I), were reported in some fish type I collagen by Kimura and others (1987). They reported that α3(I) migrated at the same position of α1(I). The elution pattern of the major collagen from the ordinary muscle was similar to that of the skin (data not shown). In the cases of yellow sea bream and tiger puffer, similar results were obtained (data not shown). As shown in Fig. 3, type I collagens were purified from skins and ordinary muscles of these 2 fish species.

The major collagens from skins and ordinary muscles of 3 fish species showed essentially similar features to carp type I collagen in amino-acid composition (Table 1), having a rela-

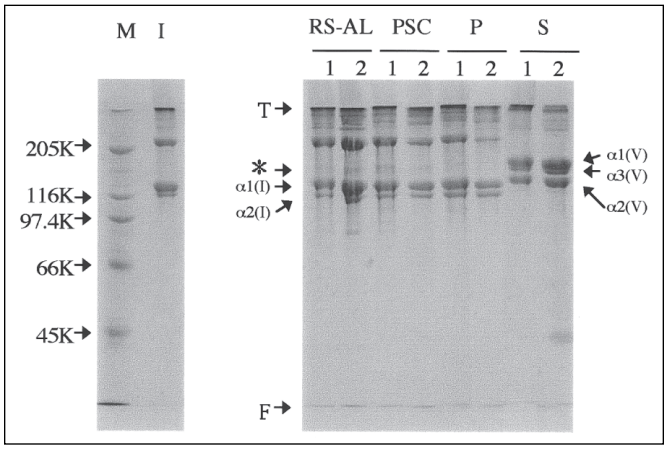

Figure 1—SDS-PAGE patterns of RS-AL, PSC, P-fraction (P), and S-fraction (S) from the skin (1) and the ordinary muscle (2) of common horse mackerel. M: molecular weight marker; I: type I collagen from carp ordinary muscle. Asterisk (*) shows the position of minor components. T = gel top and F = buffer front.

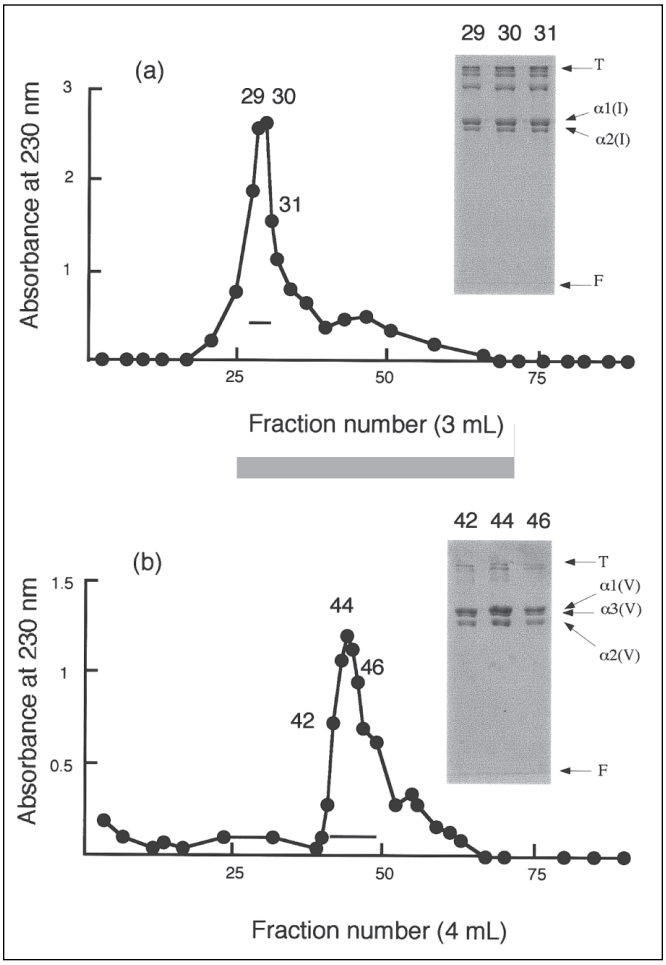

Figure 2—Phosphocellulose column chromatography of major collagen (a) and minor collagen (b) from the skin of common horse mackerel. The insets show the SDS-PAGE patterns of the fractions indicated by fraction numbers. Fractions marked by bar were collected. Letters T and F same as in Fig. 1. (a) P-fraction was dialyzed against 50 mM sodium acetate, pH 4.8, containing 0.2 M NaCl and 2 M urea and applied to phosphocellulose P-11 column (2.5 x 5 cm) that had been equilibrated with the same buffer. Elution was achieved with a linear gradient from 0.2 to 1.0 M NaCl over a total volume of 240 mL at a flow rate of 1.0 mL/min. (b) 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 x 5 cm) that 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 360 mL at a flow rate of 1.0 mL/min.

tively high content of alanine and a low content of hydroxylysine (Sato and others 1988). From these results, the major collagens in both tissues were identified as type I collagens. The contents of proline and hydroxyproline in the skin major collagens were higher and lower, respectively, than those of the muscle major collagens. Kimura and others (1988) also reported that the relative proportion of hydroxyproline/proline in muscle type I collagen was somewhat higher than that in skin type I collagen from several fish species. Kimura and others (1987) suggested that α3(I) component in some species seemed to be preferably expressed in the skin rather than other tissues. In addition, they reported the existence of α4 (I) component in eel (*Anguilla japonica*) skin. The peptide maps of the major collagens purified from the skin were similar to those of the major collagens from the ordinary muscle with slight differences (Fig. 4).

The minor collagen fraction from the skin of common horse mackerel was also further purified by phosphocellulose column chromatography (Fig. 2b). The elution pattern of the minor collagen from the ordinary muscle was similar to that of the skin collagen (data not shown). Quite similar results were also obtained for yellow sea bream and tiger puffer (data not shown). As shown in Fig. 3, type V collagens were purified from skins and ordinary muscles of these 2 fish species. SDS-PAGE patterns of the fractions in the main peak were similar to that of type V collagen comprising 3 α chains and β and γ chain sized components as reported previously (Sato and others 1989). In the present study, the positions of α chains were indicated as reported by Sato and others (1991). In the ordinary muscle of eel, Sato and others (1994) reported that type V collagen consisted mainly of 2 molecular forms of $[\alpha1(V)]_2$ α2(V) and α1(V) α3(V) α4(V), and that the relative mobility of α4(V) chain was almost identical to that of α2(V) chain by SDS-PAGE.

As shown in Table 2, the minor collagens from the skin and the ordinary muscle of 3 fish species showed similar compositional features to carp type V collagen, having a relatively low content of alanine and a high content of hydroxylysine (Sato and others 1988). From these results, minor collagens in both tissues were identified as type V collagens. But there were some differences between tissues in the content of some amino acids, such as hydroxylysine and glutamic acid. The peptide maps of the minor collagens purified from

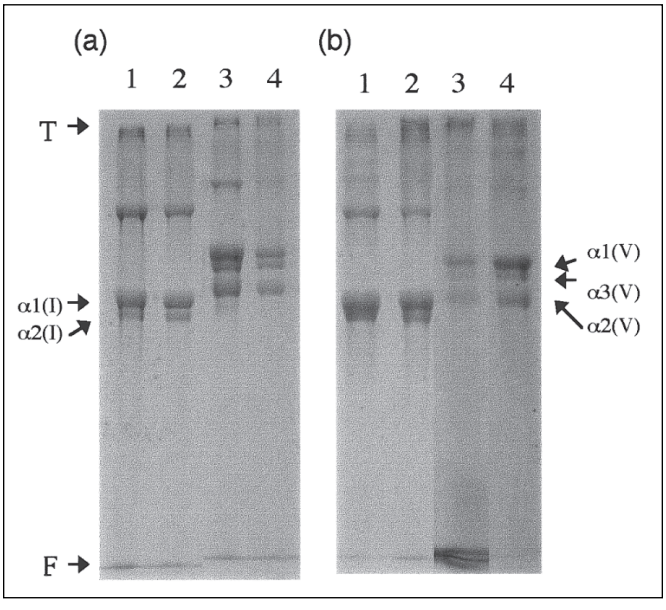

Figure 3—SDS-PAGE patterns of purified type I (1, 2), and type V (3, 4) from the skin (1, 3) and the ordinary muscle (2, 4) of yellow sea bream (a) and tiger puffer (b). T = gel top and F = buffer front.

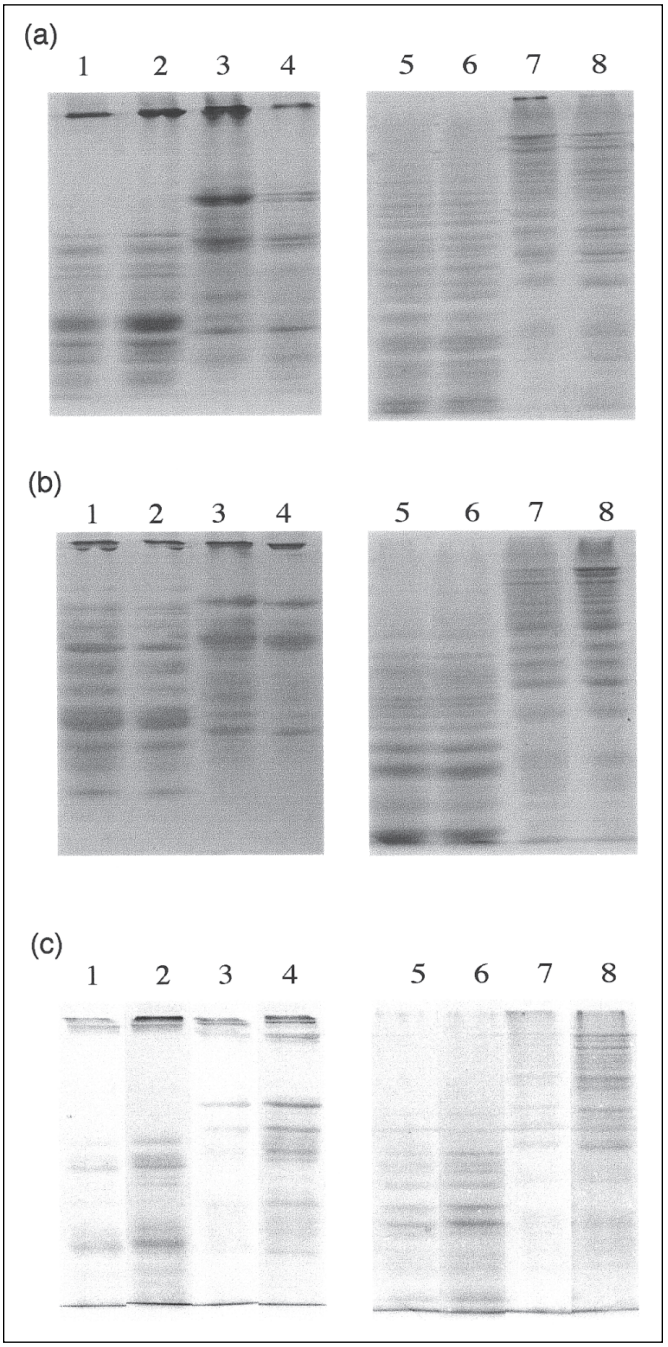

Figure 4—Peptide maps of V-8 protease (1, 2, 3, and 4) and lysyl endopeptidase (5, 6, 7, and 8) digests of collagens from the skin and the ordinary muscle of common horse mackerel (a), yellow sea bream (b), and tiger puffer (c). 1 and 5: major collagens from the skin; 2 and 6: major collagens from the ordinary muscle; 3 and 7: minor collagens from the skin; 4 and 8: minor collagens from the ordinary muscle.

the skin were similar to those of the minor collagens from the ordinary muscle with slight differences (Fig. 4).

In the present study, 2 genetically distinct types of collagen, type I and V collagens, were identified in the skin of the 3 species of fish. It was demonstrated that the distribution of molecular identity of collagens was fundamentally the same between the skin and ordinary muscle.

Type I collagen has been reported to be present in ordinary muscle (Sato and others 1988, 1989), skin (Matsui and others 1989; Kimura and others 1988), and swim bladder (Kimura and Ohno 1987) of fish. In the present study, we showed at least 2 α components of type I collagens from the skin and the ordinary muscle. We also observed the minor differences in amino-acid composition and peptide map of type I collagen between the skin and the ordinary muscle of each species. These results suggest that the relative proportion of the molecule α1(I) α2(I) α3(I) to the molecule $[\alpha 1(I)]_2$ α2(I) might be different between type I collagens from the skin and ordinary muscle.

In fish species including tiger puffer, however, type V collagen has been identified so far only in ordinary muscle (Sato and others 1989, 1997). The minor differences in amino-acid composition and peptide maps of type V collagen were observed between the skin and the ordinary muscle of each species in the present study. Some submolecular forms, as reported by Sato and others (1994) in eel muscle, might also be present in the skin and ordinary muscle of these fish species.

Conclusions

In ordinary muscle, Ando and others (1993) suggested that weakening of pericellular connective tissue caused tenderization of muscles during chilled storage. Sato and others (1997) reported that type V collagen was solubilized specifically in the softened muscle by chilled storage, while no significant changes were observed in the structure of interstitial connective tissue or biochemical properties of type I collagen.

These studies hypothesized that the solubilization of type V collagen during chilled storage might relate to the disintegration of thin collagen fibrils and weakening of the pericellular connective tissue and resultant softening of muscle. Skin type V collagen, which was identified for the 1st time in the present study, might also relate to textural changes of fish skin during chilled storage and subsequent processing. To clarify the function of collagen in connective tissue on textural changes of the skin during chilled storage or cooking, it is necessary to examine the precise concentration and localization of each collagen type in skin.

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