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Molecular species of collagen from wing muscle of skate (*Raja kenojei*)

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Abstract

Soluble collagen was prepared from skate (*Raja kenojei*) wing muscle by limited pepsin digestion. It was fractionated into two fractions, major and minor, by differential ammonium sulfate precipitation. Two collagen types were purified from the major and minor collagen fractions by phosphocellulose column chromatography and were identified as Type I and V collagens, respectively, by SDS-PAGE, peptide mapping, and amino acid analysis. © 2001 Elsevier Science Ltd. All rights reserved.

Keywords: Collagen; Skate; Muscle; Elasmobranch; Pepsin digestion

1. Introduction

Korean people have enjoyed the wing part of skate (*Raja kenojei*) as a favourite dish. Especially in the southwestern part of Korea, a traditional dish of skate is allowed to ferment in a ceramic jar without any additive over 1 week at room temperature. It is much appreciated for the elastic texture of the dermal fin rays and for a unique ammonia-like flavour derived from a large quantity of trimethylamine oxide and/or urea present (Sato, Yoshinaka, Sato, & Shimizu, 1986; Simidu & Oisi, 1951; Smith & Wright, 1999; Suyama & Suzuki, 1975). Experienced Korean cooks say that the dish, called ''Hongtak'', gets harder during fermentation. There is no toxicity to humans as long as it is kept at the recommended temperature and humidity. However, this fact seems to contradict general knowledge on storage of raw fish meat, which is softened after only 1 day in chilled storage (Ando, Toyohara, & Sakaguchi, 1992; Ando, Toyohara, Shimizu, & Sakaguchi, 1993; Hatae, Tamari, Miyanaga, & Matsumoto, 1985).

Recently, many works on fish collagen have been re-investigated. It has been reported that collagen is one of the most important components for developing raw and cooked meat texture in fish species (Hatae, Tobimatsu, Takeyama, & Matsumoto, 1986; Mizuta, Yoshinaka, Sato, & Sakaguchi, 1994, 1995; Sato et al., 1986). Although Type I collagen generally accounts for the highest proportion of total collagen, and correlates with meat texture in marine organisms, quantitatively minor amounts of other collagen types in fish have been recognized as important factors relating to postmortem tenderization under chilled storage (Ando et al., 1992; Ando, Yoshimoto, Inabu, Nakagawa, & Makinodan, 1995; Hatae et al., 1985; Sato, Ohashi, Ohtsuki, & Kawabata, 1991; Sato, Ando, Kubota, Origasa, Kawase, Toyohara et al., 1997).

Despite the recognized importance of collagens, only a few studies have been undertaken on molecular species in skate. The present paper attempts to describe the isolation and partial characterization of two genetically distinct types of collagen from skate wing muscle.

2. Materials and methods

2.1. Preparation of pepsin-solubilized collagen

All procedures were undertaken in a cold room at 5 °C. Frozen skates (*Raja kenojei*; 19 individuals; average body weight, 498 g) were transported to our

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laboratory from Kanagawa Prefecture in Japan within 24 h after death. The wing muscle, dissected from the trunk, was cut into small pieces and homogenized with 5 volumes (v/w) of 0.1M NaOH by means of a non-bubbling homogenizer (model SN-2; Nissei, Tokyo, Japan). The homogenate was then stirred overnight using a magnetic stirrer. The suspension was centrifuged at 10,000 ***g*** for 20 min. After centrifugation, 10 volumes of 0.1 M NaOH were added to the residue. The suspension was stirred overnight and centrifuged again at 10,000 ***g*** for 20min. This alkali-extraction was repeated six times in total in order to remove non-collagenous proteins effectively and to exclude the effect of endogenous proteases on collagen (Sato, Yashinaka, & Sato, 1987). The residue after alkali extraction was thoroughly rinsed with distilled water and then stirred overnight in 10 volumes of 0.5 M acetic acid. To the suspension, porcine pepsin (EC 3.4.23.1; crystallized and lyophilized, Sigma, MO) was added at an enzyme/substrate ratio of 1:20 (w/w) and digested for 24 h. After centrifugation, the supernatant was used as the pepsin-solubilized collagen preparation.

2.2. Fractionation of pepsin-solubilized collagen

The pepsin-solubilized collagen was salted out by adding NaCl to a final concentration of 2.0 M and harvested by centrifugation at 10,000 ***g*** for 20 min. The precipitate was extracted overnight with 0.5 M acetic acid containing 11%(w/v) ammonium sulfate, and then centrifuged at 10,000 ***g*** for 20 min. This procedure was repeated twice. The resultant precipitate was referred to as a major collagen fraction. At the same time, the collagen in the supernatant (minor collagen fraction) was precipitated by adding ammonium sulfate to a final concentration of 20%, and harvested by centrifugation at 10,000 ***g*** for 20 min.

2.3. Phosphocellulose chromatography

The major and minor collagen fractions were further fractionated on a column (2.5×5 cm) of phosphocellulose (P11, Whatman, Maidstone, UK) in a cold room at 5 °C. After dialysis against 20 mM Na_2HPO_4, to inactivate pepsin prior to chromatography, both major and minor collagen fractions were dialyzed against the following starting buffers; 50 mM acetic acid, pH 4.8, containing 2 M urea for the major collagen fraction, and 50 mM sodium phosphate, pH 6.8, containing 2 M urea for the minor collagen fraction. After complete dialysis against the starting buffers, samples were applied to the column. Adsorbed proteins were eluted with a linear gradient of 0–600 mM NaCl and of 0–800 mM NaCl, respectively. Elution was achieved at a flow rate of 60 ml/h. The effluent was monitored at 230 nm by a Shimadzu spectrophotometer (model UV-9900, Shimadzu, Kyoto, Japan). The appropriate fractions recovered were dialyzed against 0.5 M acetic acid containing 2.0 M NaCl, and successively against distilled water, and lyophilized.

2.4. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and peptide mapping

SDS-PAGE was performed by the method of Laemmli (1970) using 5% polyacrylamide gels at room temperature. The sample (5 μl) was applied to a sample well and electrophoresed along with molecular weight markers; myosin heavy chain (205 K, from rabbit muscle), β-galactosidase (116 K, from *Escherichia coli*), phosphorylase b (97.4 K, from rabbit muscle), albumin (66 K, from bovine serum), albumin (45 K, from hen egg; SDS-6H, Sigma). 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 and ion-exchange resin (IRA-410; Organo, Tokyo, Japan). The collagen and related peptides were stained metachromatically and non-collagenous proteins were stained orthochromatically.

Peptide-mapping with endoproteinase Glu-C from *Staphylococcus aureus* strain V-8 (V-8 protease, EC 3.4.21.19, Sigma) and lysyl endopeptidase from *Achromobacter lyticus* M 497-1 (EC 3.4.21.50, Wako, Osaka, Japan), was performed essentially as described by Cleveland, Fisher, Kirscher, and Laemmli (1977). Proteins were dissolved in 125 mM Tris–HCl, pH 6.8, containing 0.1% SDS, 1.0 mM EDTA, 0.02% bromophenol blue, and 50% glycerol. The sample (6 μl) was applied to the sample well and digested with the proteases at an enzyme/substrate ratio of 1:10 in the stacking gel for 30 min. The V-8 protease and lysyl endopeptidase digests were separated on 12.5 and 10% gels, respectively, and stained as above.

2.5. Amino acid analysis

Amino acid analysis was performed by an amino acid analysis system (Waters PICO TAG system; Waters, Milford, Mass). Samples were hydrolyzed with HCl vapour for 1 h at 150 °C in a sealed vial. The hydrolysis allows sufficient liberation of amino acids, especially aspartic acid and glutamic acid (Sato, Tsukamasa, Imai, Ohtsuki, Shimuzu, & Kawabata, 1992). Major and minor collagens of skate wing muscle were analyzed on

an ODS column (Cosmosil 5C18-AR; 4.6×250 mm; Nacalai Tesque, Kyoto, Japan).

2.6. Estimation of collagen content

The skate wing muscle (2 g) was extracted with 0.1 M NaOH to remove non-collagenous proteins and to exclude the effect of endogenous proteases on collagen (Sato et al., 1987). The residue after alkali extraction was hydrolyzed in 6 N HCl at 130 °C for 3.5 h. The hydroxyproline content in the hydrolysate was determined by the method of Woessner (1961), and the collagen content was estimated on the basis of the hydroxyproline content (7.4%) in the pepsin-solubilized major collagen, calculated from the present result of amino acid analysis.

3. Results and discussion

The approximate collagen content in the wing muscle was estimated to be 0.5% (wet tissue), 2.3% (dry tissue), and 3.6% (true protein). The collagen content (true protein) is about 1.6 times as much as that of the dry tissue owing to high contents of non-protein nitrogen such as urea, and trimethylamine oxide in the wing muscle (Sato et al., 1986; Simidu & Oisi, 1951; Smith & Wright, 1999; Suyama & Suzuki, 1975). Among these components, urea largely affects the fermentation, as is broken down by the activity of bacterial urease with the formation of ammonia (Simidu & Oisi, 1951). The collagen content per wet tissue is relatively low when compared with other elasmobranchs such as red stingray *Dasyatis akajei* (0.9%), spiny dogfish *Squalus acanthias* (1.1%), and smooth dogshark *Triakis scyllia* (2.1%; Sato et al., 1986). This suggests that the wing muscle of skate may have a relatively soft texture, according with the fact that the fishes with tender raw meat texture have lower collagen contents than those with firmer textures (Hatae et al., 1986; Sato et al., 1986), and that they are more susceptible to histological change, that is, the disintegration of the muscle between myotomes (Ando et al., 1992, 1993, 1995).

The pepsin-solubilized collagen preparations were fractionated into two fractions, major and minor, by differential ammonium sulfate precipitation. The SDS-PAGE patterns of these fractions are shown in Fig. 1. The major collagen fraction contained two bands of alpha components designated as α1 and α2, and a band of beta component. The minor collagen fraction showed three alpha bands, designated as α1, α2, and α3. Both major and minor collagen fractions showed quite similar patterns to Type I and V collagens, respectively, from carp *Cyprinus carpio* (Sato, Yoshinaka, Sato, Itoh, & Shimizu, 1988), lizardfish *Squirida elongata*, Japanese eel *Anguilla japonica*, and spotted shark *Mustelus manazo* (Sato, Yoshinaka, Itoh, & Sato, 1989), and spotted mackerel *Scomber tapeinocephalus* (Sato, Yoshinaka, Sato, & Tomita, 1989).

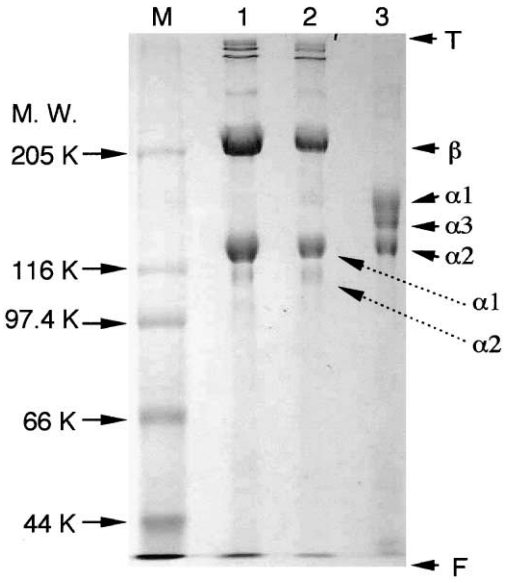

Fig. 1. SDS-PAGE patterns (5% gel) of pepsin-solubilized collagen (lane 1), major (lane 2) and minor (lane 3) collagen fractions from the skate wing muscle, along with the molecular weight standards (lane M). The arrows M.W. designate the molecular weights of standard proteins, myosin heavy chain (205 K, from rabbit muscle), β-galactosidase (116 K, from *Escherichia coli*), phosphorylase b (97.4 K, from rabbit muscle), albumin (66 K, from bovine serum), and albumin (45 K, from hen egg). Letters T and F show the top and buffer front of gel, respectively.

The major and minor collagen fractions were further fractionated by phosphocellulose column chromatography under different conditions (Figs. 2 and 3). The major and minor collagen fractions showed a single peak with a small shoulder. The pooled fractions underlined A and B were recovered as purified major and minor collagens, respectively. Fig. 4 shows the peptide maps of lysyl endopeptidase and V-8 protease digests of major and minor collagens from the skate wing muscle. The major collagen showed quite distinct patterns from those of the minor collagen. Table 1 shows the results of amino acid analysis of the purified major and minor collagen types, compared with Type I and V collagens from the muscle of spotted shark (Sato et al., 1989). Major and minor collagens showed quite similar composition to those of Type I and V collagens from the muscle of spotted shark. The amino acid composition of the minor collagen was different from that of the major collagen, especially in the higher levels of hydroxylysine and leucine, and the lower level of alanine. These data are also quite similar to those for Type V collagen from the spotted shark muscle *Mustelus manazo* (Sato et al., 1989) which shows typical compositional features of mammalian and avian Type V collagen except for a lower content of hydroxyproline, owing to their body temperature difference, that is, the

Table 1
Amino acid composition of major and minor collagens from wing muscle of skate compared with Type I and V collagens from muscle of spotted shark (residues/1000 residues)

	Skate (*Raja kenojei*)[a]		Spotted shark (*Mustelus manazo*)[b]	
	Major	Minor	I	V
Hydroxyproline	74	84	76	82
Aspartic acid	36	43	35	42
Threonine	36	39	27	31
Serine	46	41	49	46
Glutamic acid	78	93	75	97
Proline	83	98	98	95
Glycine	356	348	341	324
Alanine	115	60	109	62
Half-cysteine	0	1	0	0
Valine	25	21	28	25
Methionine	9	6	12	9
Isoleucine	17	18	16	22
Leucine	22	38	26	40
Tyrosine	2	4	2	6
Phenylalanine	12	12	13	15
Hydroxylysine	6	22	7	26
Lysine	25	19	27	21
Histidine	8	9	8	9
Arginine	51	44	52	48

[a] Data show the means of three determinations for the identical collagen samples.
[b] Sato et al. (1989).

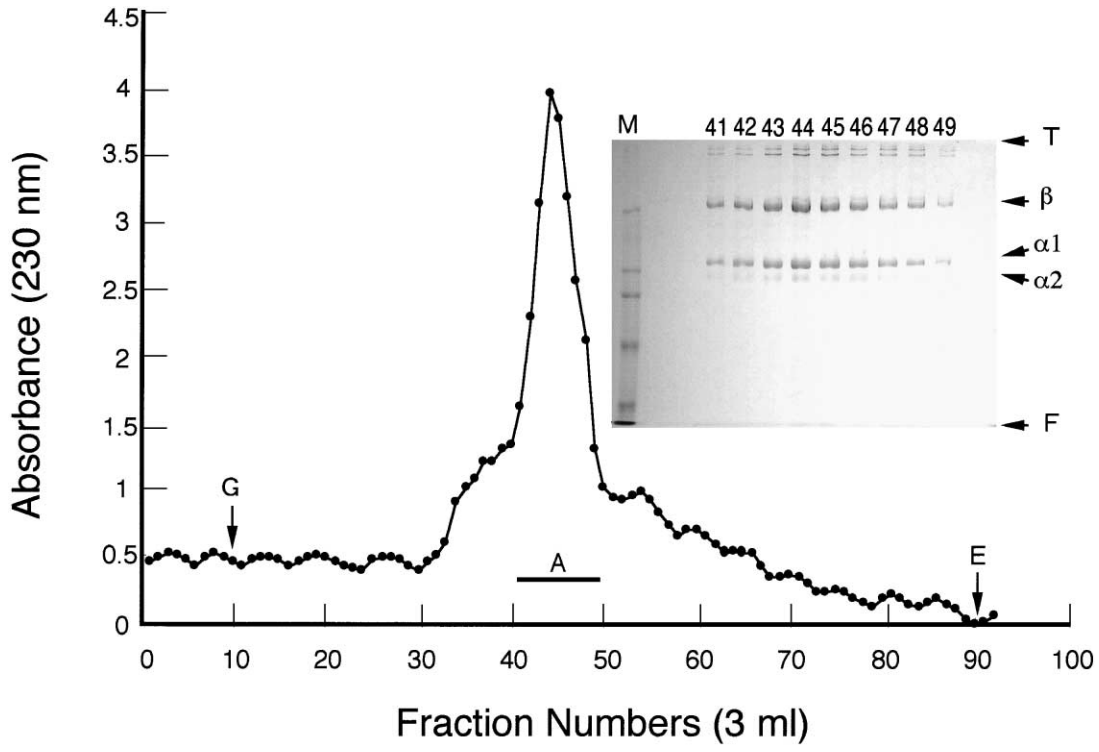

Fig. 2. Phosphocellulose column chromatography of the major collagen fraction. The column (2.5×5cm) was equilibrated with 50 mM sodium acetate, pH 4.8, containing 2 M urea. Adsorbed proteins were eluted with a linear gradient of 0–600 mM NaCl over a total volume of 240 ml at a flow rate of 60 ml/h. The inset shows the pattern of SDS-PAGE (5% gel) of the fractions from No. 41 to 49, along with the molecular weight standard (lane M). Letters G and E show the beginning and ending breakpoints of a linear gradient 0–600 mM NaCl, respectively. Letters T and F are the same as in Fig. 1.

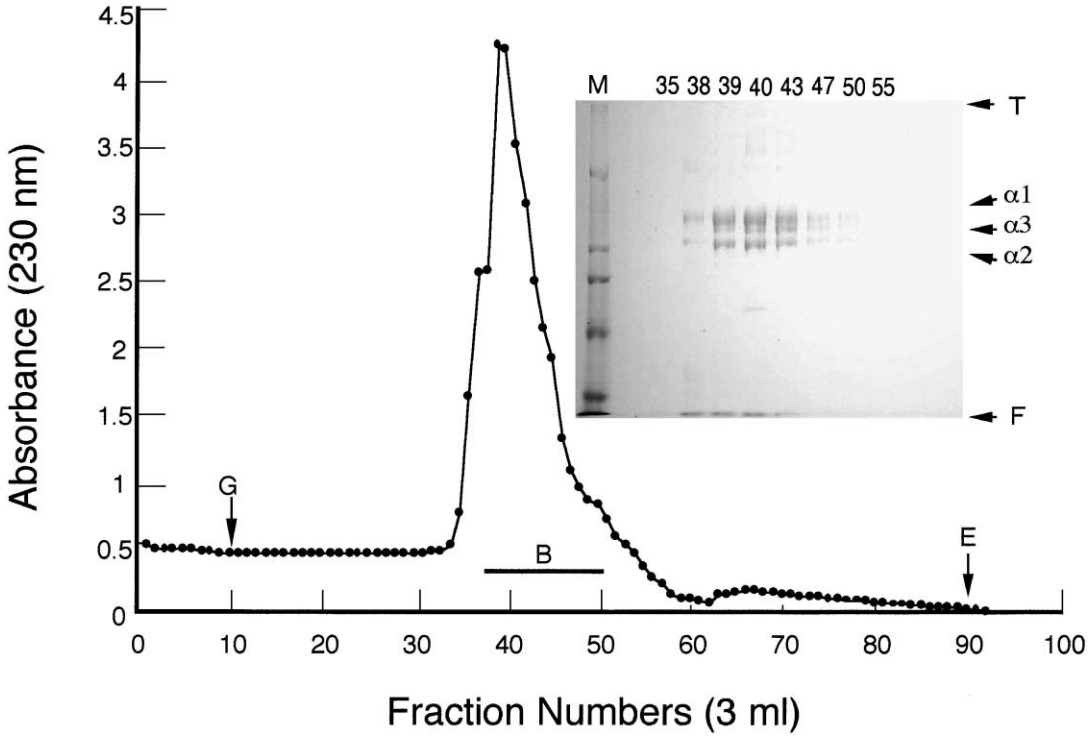

Fig. 3. Phosphocellulose column chromatography of the minor collagen fraction. The column (2.5×5cm) was equilibrated with 50 mM sodium phosphate, pH 6.8, containing 2 M urea. Adsorbed proteins were eluted with a linear gradient of 0–800 mM NaCl over a total volume of 240 ml at a flow rate of 60 ml/h. The inset shows the pattern of SDS-PAGE (5% gel) of the fractions from No. 38 to 50, along with the molecular weight standard (lane M). Letters G and E show the beginning and ending breakpoints of a linear gradient 0–800 mM NaCl, respectively. Letters T and F are the same as in Fig. 1.

denaturation temperature of collagen depends on the content of hydroxyproline (Takahashi & Yokoyama, 1954). These combined results suggested that the major and minor collagens were genetically different from each other and may be classified as Type I and V collagens, respectively.

In the present study, two types of collagen, Type I and V, were isolated from the wing muscle of skate, by differential salt precipitation and phosphocellulose column chromatography. Based on the swimming mode for flapping its sail-like wings with a ripping motion passing along the fins from front to rear, the wing muscle of skate was expected to contain a large amount of collagen, because the larger the movement of the body by swimming, the higher the collagen content in that part is. (Sato et al., 1986; Yoshinaka, Sato, Anbe, Sato, & Shimizu, 1988). However, there was a relatively small amount of collagen in the skate wing muscle, unexpectedly, as compared with other elasmobranchs. Interestingly, the minor collagens were higher than the major collagen in the contents of amino acids, proline and hydroxyproline, as well as aspartic and glutamic acids. This strongly indicated another possibility, that the minor collagen may have higher thermal stability, owing to hydrogen bonds, with these amino acids participating in the development of thermal stability (Arai, 1991; Harrington, 1964; Takahashi & Yokoyama, 1954; Zhu

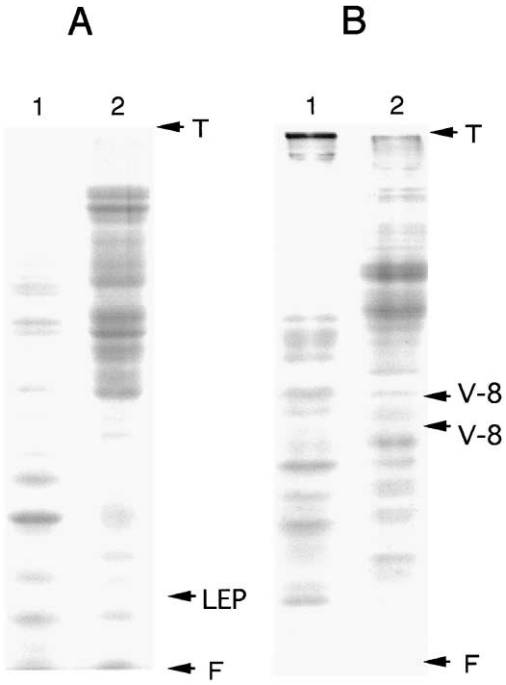

Fig. 4. Peptide maps of lysyl endopeptidase (A, 10% gel) and V-8 protease (B, 12.5% gel) digests of major(1) and minor(2) collagens from the skate wing muscle. The arrows, LEP and V-8, show the positions of the enzyme components of lysyl endopeptidase and V-8 protease, respectively. Letters T and F are the same as in Fig. 1.

& Kimura, 1991). Further studies on the wing part of skate are now in progress in order to more clearly elucidate the contribution of collagen to its texture.

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