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Rapid communication

Purification and characterization of molecular species of collagen in the skin of skate (*Raja kenojei*)

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Abstract

Two types, one major and the other minor, of collagen were obtained from pepsin-solubilized collagen of the skate (*Raja kenojei*) skin. The major collagen was identified as Type I collagen because of a similar characteristic to those of Type I collagens from the skate muscle and cartilage. On the other hand, the SDS–PAGE pattern and peptide maps of the minor collagen were different from those of Type V and XI collagens of the skate muscle and cartilage, respectively. However, the minor collagen was similar, in amino acid composition, to Type V collagen from the skate muscle. These results suggest the existence of a molecular subspecies of Type V collagen in skate skin which is different from the muscle Type V collagen in primary structure or subunit composition.

Keywords: Fish; Elasmobranch; Skate; Collagen; Skin; Type I; Type V; Denaturation temperature

1. Introduction

In the processing of most commercial fishes, the skin is dumped as waste, and can cause pollution and offensive odours. From the viewpoint of environmental protection, as well as utilization of limited marine bioresources, it is important to develop a preparation method of fish skin collagen for making more effective use of this under-utilized resource or waste material, and to suggest its availability as a substitute for mammalian collagen in foods, cosmetics and industrial materials.

Skate is an important commercial fish in the southern part of Korea, especially in the Joellanam-do Province. The skinned disc of the skate is mainly processed to a fermented product, Hongtak, but the skin is dumped without utilization. For making more effective use of the dumped skin as a collagen resource, it is necessary to obtain fundamental information about the skin collagen of the skate .

Among the elasmobranchs, which comprise sharks, skates and rays, there are some studies regarding the biochemical properties of Type I collagen from shark skin (Kawaguchi, 1985; Kimura, Kamimura, Takema, & Kubota, 1981; Lewis & Piez, 1964; Motta, 1977; Nomura, Yamano, & Shirai, 1995; Nomura, Yamano, Hayakawa, Ishii, & Shirai, 1997; Yoshimura, Terashima, Hozan, & Shirai, 2000). However, there is still only limited information on the molecular species of collagen in shark skin. On the other hand, no biochemical information on collagen from the skate skin has so far been reported. The present paper describes the biochemical characteristics of two genetically different collagens in the skin of the skate, *Raja kenojei*.

2. Materials and methods

2.1. Materials

Skate (*Raja kenojei*) (body weight, 450–500 g) were obtained alive from local fishermen in Obama, Fukui, Japan. Rainbow trout (*Oncorhynchus mykiss*) (body weight, 550–570 g) were purchased from a local nursery

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Table 1
Amino acid composition of Type I and V collagens from skate skin with comparison of Type I and V collagens from the skate muscle (residues/1000 residues)

	Skate (*Raja kenojei*)[a]			
	Skin		Muscle[b]	
	I	V	I	V
Hydroxyproline	72	83	74	84
Aspartic acid	37	43	36	43
Threonine	36	38	36	39
Serine	46	39	46	41
Glutamic acid	75	93	78	93
Proline	81	98	83	98
Glycine	348	344	356	348
Alanine	114	56	115	60
Half-cystine	1	0	0	1
Valine	27	21	25	21
Methionine	16	11	9	6
Isoleucine	17	18	17	18
Leucine	23	39	22	38
Tyrosine	2	3	2	4
Phenylalanine	12	13	12	12
Hydroxylysine	7	29	6	22
Lysine	28	19	25	19
Histidine	9	10	8	9
Arginine	49	44	51	44

[a] Data show the means of three determinations for the identical collagen samples.
[b] Mizuta et al. (2002).

in Mihama, Fukui, Japan. The skin of the skate and the muscle of the rainbow trout were dissected out from the bodies and stored at −80 °C until used (see Table 1).

2.2. Preparation of acid-soluble collagen

All procedures were performed in a cold room at 5 °C. The skin of the skate and the muscle of the rainbow trout were cut into small pieces and then homogenized with 10 volumes (v/w) of 0.1 M NaOH and extracted for 24 h. The alkali-extraction was done to remove non-collagenous proteins effectively and to exclude the effect of endogenous proteases on collagen, as described previously (Yoshinaka et al., 1985). The residues after alkali extraction (RS-AL) were thoroughly rinsed with distilled water and then stirred overnight in 10 volumes of 0.5 M acetic acid. The suspensions were centrifuged at 10,000*g* for 20 min. The resultant supernatants were successively dialyzed against distilled water and lyophilized and were designated as acid-soluble collagen (ASC).

2.3. Preparation and fractionation of pepsin-solubilized collagen

The RS-AL from the skate skin was digested with porcine pepsin (EC 3.4.23.1; crystallized and lyophilized, Sigma, MO) in 0.5 M acetic acid at an enzyme/substrate ratio of 1:20 (w/w) and digested for 24 h at 5 °C. After centrifugation (10,000*g*, 20 min), the supernatant was used as a pepsin-solubilized collagen (PSC) preparation. The differential salt precipitation of the PSC was conducted as described previously (Mizuta, Hwang, & Yoshinaka, 2002, 2003). The resultant precipitate and supernatant were designated as P-11 and S-11 fractions, respectively.

2.4. Phosphocellulose column chromatography

Two fractions (P-11 and S-11) of the PSC were further purified by phosphocellulose column chromatography, according to the method reported by Mizuta et al. (2002). The recovered fractions were dialyzed against distilled water and lyophilized. The resultant fractions were referred to as P-11 and S-11 collagens, respectively.

2.5. Analytical methods

SDS–PAGE was performed by the method of Laemmli (1970), using 5% polyacrylamide gels. Gels were stained for protein with Coomassie Brilliant Blue (CBB) R-250, essentially as described by Fairbanks, Steck, and Wallach (1971). The collagen and related peptides were stained metachromatically and non-collagenous proteins were stained orthochromatically.

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

Amino acid analysis and collagen content determination were conducted as described previously (Mizuta et al., 2002). The collagen content was estimated on the basis of the hydroxyproline content (7.2%) in the P-11 collagen, calculated from the present result of amino acid analysis.

2.6. Determination of thermal stability

The ASCs were prepared from the rainbow trout muscle and skate skin as described above, and the ASC from rat tail tendon (BD Biosciences, USA) was purchased through a local reagent dealer. Each ASC (0.7 mg) was dissolved in 0.7 ml of 0.1 M acetic acid, and the solution was applied to a differential scanning calorimeter (micro DSC III; Setaram, France). All thermograms were recorded at a constant heating rate (0.5 °C/min). The denaturation temperature was determined by using software provided by the instrument's manufacturer.

3. Results and discussion

The approximate collagen content in the skin tissue was estimated to be 8.9% and 35.6% of wet and dry tissue, respectively. The collagen content in the skin is much higher than those of the muscle and the cartilage reported previously (Mizuta et al., 2002, Mizuta, Hwang, & Yoshinaka, 2003). As the skate belongs to the elasmobranchs, the skin may play an important role, since elasmobranchs have

cartilaginous skeletons and thick skin instead of the bone and scale of the teleosts in order to maintain their body form and to avoid the degradation of muscle structure (Motta, 1977). The data mentioned above also indicate that inedible skate skin is available for marine collagen resources.

The major part of the collagen in the skate skin was readily soluble in dilute acetic acid. This is in contrast to land vertebrate collagen which is hard to dissolve in acetic acid without pepsin digestion (Bracho & Harrd, 1990). The high acid-solubility of the skate skin collagen was similar to those of the skin collagens from other fishes, such as the blue shark *Prionace glauca*, the blue grenadier *Macruronus novaezelandiae* and the Pacific lingcod, *Ophiodon elongatus*. These facts may result from lower collagen cross-linking in the fish collagen than in land vertebrate collagen (Bracho & Harrd, 1995; Kawaguchi, 1985; Ramshow, Werkmeister, & Bremner, 1988). The denaturation temperatures of ASCs from the skate, the rainbow trout and the rat were determined (Fig. 1). Investigations on the stability of the native collagen molecule deal with processes associated with the collagen-gelatin transition (Pikkarainen, 1968). As expected, the fish collagens (Fig. 1, ○ and ●) have denaturation temperature values more than 12 °C lower than the rat collagen (Fig. 1, ▲), indicating that a low denaturation temperature may be due to a low degree of proline hydroxylation in the fish collagens. Moreover, the skin ASC from the skate exhibited more resistance to heat-denaturation than did the muscle ASC from the rainbow trout; the former has a denaturation temperature of 24.9 °C, which is significantly higher, by about 3.9 °C, than that of the latter. The result also suggested that the thermal stability could be directly related to the degree of proline hydroxylation of collagens of skate and rainbow trout (Table 2). Similar results were previously obtained for other fishes (Kimura, Zhu, Matsui, Shijoh, & Takamizawa, 1988).

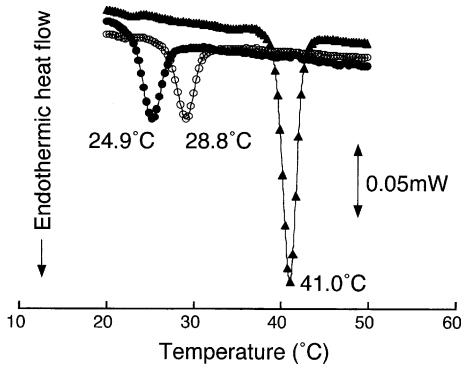

Fig. 1. Thermal transition curves of acid-soluble collagens from skate skin (○), rainbow trout muscle (●) and rat tail tendon (▲). The denaturation temperature of the soluble collagens (1 mg/ml) was measured by a differential scanning calorimeter. All thermograms were recorded at a constant heating rate (0.5 °C/min). The individual denaturation temperature was determined by using software provided by the instrument's manufacturer. The denaturation temperature is shown on each thermogram with the mean of three determinations for the identical samples.

The pepsin-solubilized collagen preparation was fractionated into major (P-11) and minor (S-11) fractions by differential ammonium sulfate precipitation. The P-11 and S-11 fractions were further purified by phosphocellulose column chromatography (Figs. 2 and 3). The underlined fractions were recovered as purified P-11 and S-11 collagens, respectively. The SDS–PAGE patterns of these collagens are shown in Fig. 4, together with collagens from the muscle and cartilage described previously (Mizuta et al., 2002, 2003). The SDS–PAGE pattern of P-11 collagen (lane 6) showed two bands of an α component, designated as α1 and α2, and a band of a β component. This pattern was similar to those of Type I collagens from the muscle (lane 1) and cartilage (lane 3). In addition, a faint band of the α2 component and a dense band of the β component suggest that the α2 component may dimerize into the β component and form β_{12} dimer. Similar results were previously reported for other elasmobranch Type I collagens from the great blue shark *Prionace glauca* and the dogfish shark *Squalus acanthias* (Kimura et al., 1981; Lewis

Table 2
Thermal stability and degree of proline hydroxylation of muscle and skin Type I collagens

	Denaturation temp (°C)		Degree of proline hydroxylation (%)	
	Muscle	Skin	Muscle	Skin
Skate	ND[a]	28.8	47.1[b]	47.1
Rainbow trout	24.9	ND[a]	39.6[c]	39.8[c]

[a] Not determined.
[b] Mizuta et al. (2002).
[c] Saito et al. (2001).

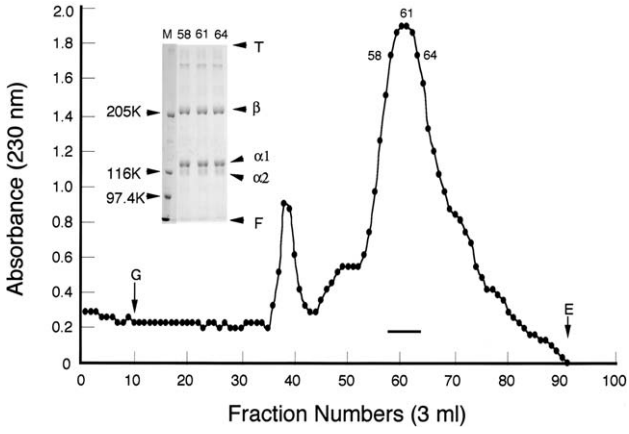

Fig. 2. Phosphocellulose column chromatography of the P-11 fraction. The column (2.5 × 5 cm) was equilibrated with 50 mM sodium acetate, pH 4.8, containing 2 M urea. Adsorbed proteins were eluted with a linear gradient of 0–600 mM NaCl over a total volume of 240 ml at a flow rate of 60 ml/h. Letters G and E show the beginning and ending breakpoints of the linear gradient 0–600 mM NaCl, respectively. The inset shows the SDS–PAGE pattern (5% gel) of the fractions indicated by the numbers 58, 61, 64. Lane M designates 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). Letters T and F show the top and buffer front of the gel, respectively.

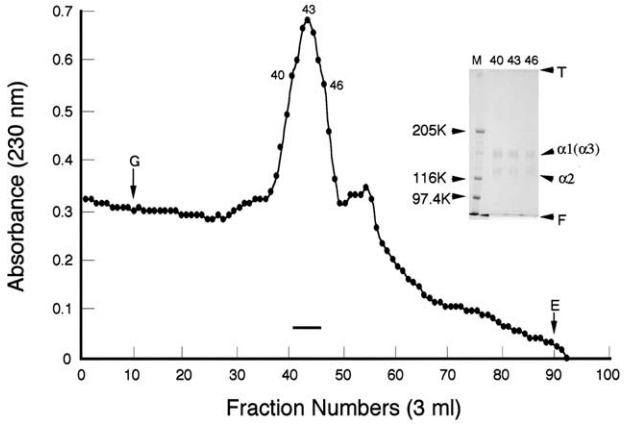

Fig. 3. Phosphocellulose column chromatography of the S-11 collagen fraction. The column (2.5 × 5 cm) was equilibrated with 50 mM sodium phosphate, pH 6.8, containing 2 M urea. Adsorbed proteins were eluted with a linear gradient of 0–800 mM NaCl over a total volume of 240 ml at a flow rate of 60 ml/h. Letters G and E show the beginning and ending breakpoints of the linear gradient 0–800 mM NaCl, respectively. The inset shows SDS–PAGE pattern (5% gel) of the fractions indicated by the numbers 40, 43, 46, along with the molecular weight standard (lane M). Letters M, T and F are the same as in Fig. 2.

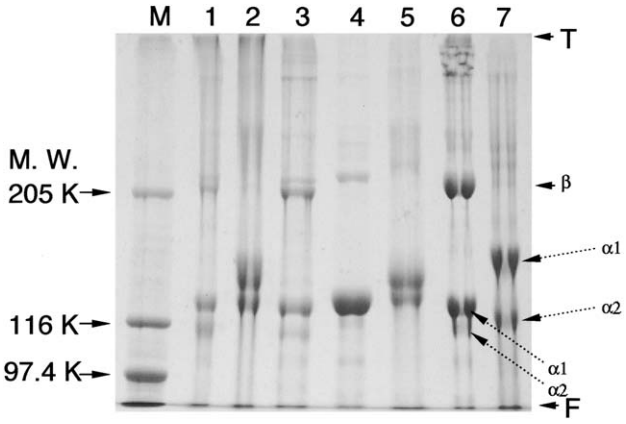

Fig. 4. SDS–PAGE patterns (5% gel) of P-11 (lane 6) and S-11 (lane 7) collagens from the skate skin with comparison of Type I (lane 1) and Type V (lane 2) collagens from the muscle (Mizuta et al., 2002), Type I (lane 3), Type II (lane 4) and Type XI (lane 5) collagens from the cartilage (Mizuta et al., 2003), respectively. Letters M, T and F are the same as in Fig. 2.

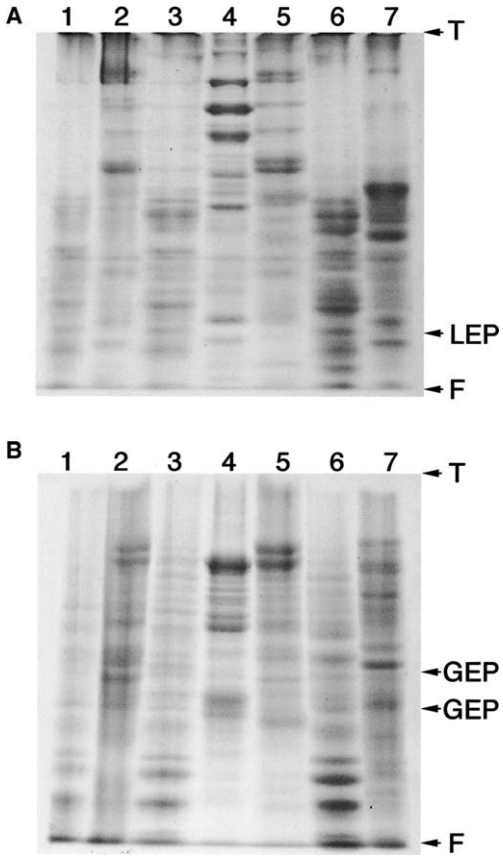

Fig. 5. Peptide maps of lysyl endopeptidase (A, 10% gel) and glutamyl endopeptidase (B, 12.5% gel) digests of various skate collagens. The lanes are the same as in Fig. 4. The arrows LEP and GEP show the positions of the enzyme components of lysyl and glutamyl endopeptidase, respectively. Letters T and F are the same as in Fig. 2.

& Piez, 1964). The P-11 collagen showed quite similar peptide maps (Fig. 5) and amino acid compositions, especially in the high level of alanine (Table 1), to the Type I collagen from the muscle described previously (Mizuta et al., 2002). These combined results indicate that P-11 collagen should be identified as Type I collagen.

As shown in Fig. 4, the S-11 collagen (lane 7), representing two α bands, designated as α1 and α2, showed quite a different SDS–PAGE pattern from Type V (lane 2) and Type XI (lane 5), collagens from the muscle and cartilage, respectively. However, the results are different from Type V collagens from some teleosts, such as the common horse mackerel *Trachurus japonicus*, the yellow sea bream *Dentex tumifroms* and the tiger puffer *Takifugu rubripes*, demonstrating that the distribution of molecular identity of collagens is fundamentally the same between skin and muscle (Yata, Yoshida, Fujisawa, Mizuta, & Yoshinaka, 2001). In addition, peptide maps of the skin S-11 collagen are quite different from those of Type V and XI collagens in the skate muscle and cartilage (Fig. 5), and also different from those of the Type V collagens in the skin and muscle of some teleosts (Yata et al., 2001). From the amino acid analysis (Table 1), S-11 collagen showed quite similar composition to that of Type V collagen from the muscle described previously Mizuta et al. (2002). The amino acid composition of the S-11 collagen is different from that of the Type I collagen in the high contents of hydroxylysine and leucine and the low content of alanine. These combined results suggest that the S-11 collagen should be classified as a molecular subspecies of Type V collagen, present in the skate skin as a minor collagen. In the present study, it is suggested that the collagen from the skate skin could be utilized as a marine collagen resource in view of its high solubility, high collagen content, and relatively high heat stability. Further studies are in progress to determine the primary structure of Type V/XI procollagen α chains, in order to elucidate the difference between the minor collagens from the skin and muscle of the skate.

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