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Molecular species of collagen in pectoral fin cartilage of skate (*Raja kenojei*)

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Abstract

Soluble collagen was prepared from the pectoral fin cartilage of skate (*Raja kenojei*) by limited pepsin digestion. It was fractionated into three fractions by differential salt precipitation. All collagen fractions were further purified by phosphocellulose column chromatography and were characterized with respect to solubility, mobility on SDS–PAGE, peptide map, and amino acid composition. The resultant data indicate the distribution of three molecular species of collagen, corresponding to Type I and II as major collagens and Type XI as a minor collagen, respectively.

Keywords: Collagen; Elasmobranch; Skate; Cartilage; Pepsin digestion; Type I; Type II; Type XI

1. Introduction

Hongtak is an indigenous fermented product in the Cheonnam Province of Korea, made mainly from the pectoral fin of skate, *Raja kenojei*. The fermentation is usually carried out in a ceramic jar without any additive over 1 week at room temperature, in contrast to salted and fermented products, such as Heshiko in Japan (Itou & Akahane, 2000) and Jeotkal in Korea (Steinkraus, 1983). In addition, the Hongtak has two main characteristics; one is an ammonia-like odour which is produced in the process from nitrogen components such as trimethylamineoxide and urea, decomposing to trimethylamine and ammonia, and the other is an elastic texture of dermal fin rays, possibly developed by macromolecules, including collagen and proteoglycan (Bailey & Light, 1989; Haard & Simpson, 2000; Hatton & Gibb, 1999; Kanoh, Watabe, Takewa, & Hashimoto, 1985; Konosu, Watanabe, & Shimizu, 1974; Smith & Wright, 1999; Watabe, Ochiai, Kanoh, & Hashimoto, 1983).

Recently, it has been reported that collagen is one of the most important components for developing raw or cooked meat texture in several aquatic animals (Mizuta, Yamada, Miyagi, & Yoshinaka, 1999; Mizuta, Yoshinaka, Sato, & Sakaguchi, 1994, 1997; Olaechea, Ushio, Watabe, Takeda, & Hatae, 1993; Sato et al., 1997; Sato, Yoshinaka, Sato, & Shimizu, 1987). But, there is still limited information on the molecular species of collagen in the cartilage of elasmobranch fish, except for some studies regarding the biochemical properties of shark collagen, mainly focusing on Type I collagen from skin (Kimura, Kamimura, Takema, & Kubota, 1981; Motta, 1977; Nomura, Yamano, Hayakawa, Ishii, & Shirai, 1997; Nomura, Yamano, & Shirai, 1995; Yoshimura, Terashima, Hozan, & Shirai, 2000). Furthermore, no minor collagen has ever been isolated though the existence of Type I and II collagens was reported in the cartilage of the shark *Carcharius acutus* (Rama & Chandrakasan, 1984; Sivakumar & Chandrakasan, 1998). Therefore, the present study describes the isolation and partial characterization of collagen types in pectoral fin cartilage of skate, including a minor collagen.

2. Materials and methods

2.1. Preparation of pepsin-solubilized collagen

All procedures were done in a cold room at 4 °C, and all centrifugations were undertaken at 10,000*g* for 20 min. Skates (*Raja kenojei*; 19 individuals; average body

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weight, 498 g), frozen within 24 h after death, were obtained from a fisherman in Kanagawa Prefecture, Japan. Cartilage embedded in pectoral fin was dissected out from the trunk using a knife, then attached muscle and perichondrium were removed thoroughly by means of brushing and suspending in five volumes of 0.1 M NaOH. The cartilage was extracted with 0.1 M NaOH twice, after cutting into small pieces (about 2 mm^2) in order to remove non-collagenous materials effectively and to exclude the effect of endogenous proteases on collagen (Sato et al., 1987). The residue after the alkali extraction was rinsed thoroughly with distilled water and was treated with three changes of 0. 5 M EDTA solution, the pH of which was adjusted to 8.0 with NaOH, during 12 days (Herring, 1976; Rama & Chandrakasan, 1984). After centrifugation, the precipitate was washed thoroughly with distilled water and then with 50 mM Tris–HCl, pH 7.4, containing 1 M NaCl for 3 days (Rama et al., 1984). Finally, it was washed thoroughly with distilled water again. The resultant pellet was digested for 24h with porcine pepsin (EC 3.4.23.1; crystallized and lyophilized, Sigma, MO) at an enzyme/substrate ratio of 1:20 (w/w) in 10 v of 0.5M acetic acid. After centrifugation, the supernatant was used for the pepsin-solubilized collagen preparation.

2.2. Fractionation of pepsin-solubilized collagen

The pepsin-solubilized collagen preparation was salted out by adding NaCl to a final concentration of 2.0 M. The precipitate was extracted overnight with 0.5 M acetic acid containing 11% (w/v) ammonium sulphate, and then centrifuged. This procedure was repeated twice. The resultant precipitate (P-11 fraction) was further fractionated by differential NaCl precipitation at neutral pH. The P-11 fraction was dissolved in 50 mM Tris–HCl, pH 7.5, containing 2.2 M NaCl and then dialyzed thoroughly against the same buffer. The precipitated collagen was collected by centrifugation and was referred to as P-2.2 fraction. Solid NaCl was added to the supernatant to a final concentration of 4.0 M. The resultant precipitate was harvested by centrifugation and was referred to as S-2.2 fraction. At the same time, the soluble collagen fraction, at 11% ammonium sulphate (S-11 fraction), was precipitated by adding ammonium sulphate to a final concentration of 20%, and harvested by centrifugation.

2.3. Phosphocellulose chromatography

All fractions were further purified by phosphocellulose (P11, Whatman, Maidstone, UK) column chromatography. After dialyzing completely against 50 mM Tris–HCl containing 2 M urea, pH 8.6, to inactivate pepsin prior to chromatography, all fractions were dialyzed thoroughly against the following starting buffers; 50mM acetic acid, pH 4.8, containing 2 M urea for the P-2.2 and S-2.2 fractions, and 50 mM sodium phosphate, pH 6.8, containing 2 M urea for the S-11 fraction, respectively. After dialysis, each sample was applied to the column. Adsorbed proteins were eluted at a flow rate of 60 ml/h with a linear gradient of 0–600 mM NaCl for the P-2.2 and S-2.2 fractions and of 0–800 mM NaCl for the S-11 fraction, respectively. The effluent was monitored at 230 nm by a Shimadzu spectrophotometer (model UV-9900, Shimadzu, Kyoto, Japan). The appropriate fractions were pooled, dialyzed against 0.5 M acetic acid containing 2.0 M NaCl, and successively against distilled water, and then lyophilized.

2.4. Analytical methods

SDS–PAGE was performed by the method of Laemmli (1970), using 5% polyacrylamide gel at room temperature. Gels were stained for protein with Coomassie Brilliant Blue R-250 according to the method reported previously (Mizuta, Hwang, & Yoshinaka, 2002). The collagen and related peptides were stained metachromatically and non-collagenous proteins were stained orthochromatically (Duhamel, 1983; Micko & Schlaepfer, 1978)

Peptide mapping with V-8 protease 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 using 12.5 and 10% gels, respectively, as reported previously (Mizuta et al., 2002).

Amino acid analysis was performed by an amino acid analysis system (Waters PICO TAG system; Waters, Milford, Mass). Samples were hydrolyzed with HCl vapor for 1 h at 150 °C in a sealed vial. Amino acid derivatives were analyzed on an ODS column (Cosmosil 5C18-AR; 4.6×250mm; Nacalai Tesque, Kyoto, Japan).

The collagen content was determined essentially as previously described (Mizuta et al., 2002). The cartilage tissue was extracted with 0.1 M NaOH to remove non-collagenous substances and to exclude the effect of endogenous proteases on collagen (Sato et al., 1987). The residue after alkali extraction was washed thoroughly with cold-distilled water and then lyophilized. The lyophilized sample (0.2 g) was hydrolyzed in 6N 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 (7.5%) in the pepsin-solubilized major collagen (S-2.2 fraction, Type II fraction), calculated from the present result of amino acid analysis.

3. Results and discussion

3.1. Fractionation of collagen types

The approximate collagen content in the cartilage tissue of pectoral fin was estimated to be 1.7 and 31.1% of wet and dry tissue, respectively. The collagen content of wet tissue was about three times as much as that of wing muscle (Mizuta et al., 2002). Moreover, the collagen content of dry tissue was even higher than that of cranial cartilage of squid *Todarodes pacificus* (23%) that has a strong resemblance in morphological and histological properties to vertebrate hyaline cartilage (Kimura & Karasawa, 1985; Philpott & Person, 1970). Therefore,

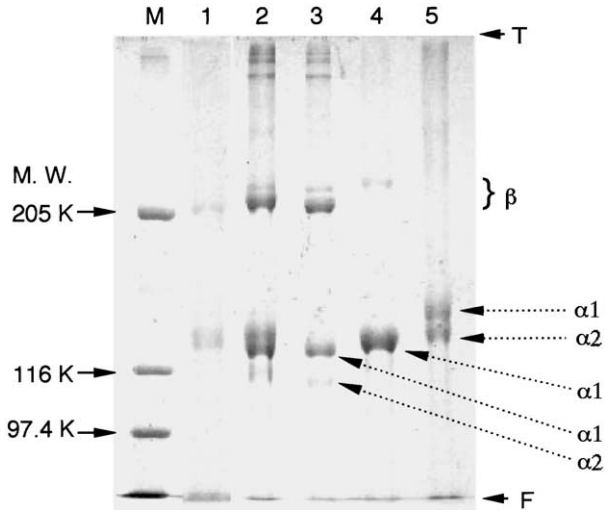

Fig. 1. SDS–PAGE patterns (5% gel) of pepsin-solubilized collagen (lane 1), P-11 (lane 2), P-2.2 (lane 3), S-2.2 (lane 4), and S-11 (lane 5) fractions from the pectoral fin cartilage of skate, along with the molecular weight standards (lane M). The arrows M.W. designate the molecular weights of standard proteins, myosin heavy chain (205k, from rabbit muscle), β-galactosidase (116k, from *Escherichia coli*), and phosphorylase *b* (97.4k, from rabbit muscle). Letters T and F show the top and buffer front of the gel, respectively.

Table 1
Amino acid composition of cartilaginous collagens from skate pectoral fin compared to Type I and V collagens from skate wing muscle (residues/1000 residues)

	Skate (*Raja kenojei*)[a]				
	Cartilage			Muscle[b]	
	I	II	XI	I	V
Hydroxyproline	74	75	74	74	84
Aspartic acid	36	41	43	36	43
Threonine	37	39	36	36	39
Serine	45	30	30	46	41
Glutamic acid	78	90	98	78	93
Proline	83	113	103	83	98
Glycine	354	352	353	356	348
Alanine	115	98	63	115	60
Half-cysteine	0	0	0	0	1
Valine	25	19	22	25	21
Methionine	10	10	11	9	6
Isoleucine	18	8	16	17	18
Leucine	22	27	39	22	38
Tyrosine	2	2	4	2	4
Phenylalanine	12	12	12	12	12
Hydroxylysine	6	18	26	6	22
Lysine	26	12	14	25	19
Histidine	8	2	9	8	9
Arginine	50	51	47	51	44

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

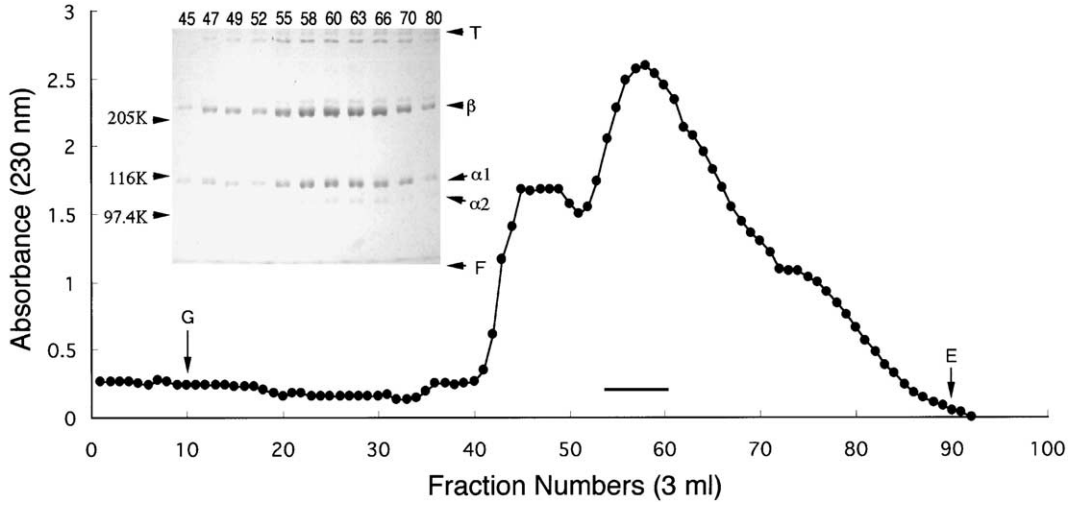

Fig. 2. Phosphocellulose column chromatography of the P-2.2 fraction. The column (2.5×5 cm) was equilibrated with 50 mM sodium acetate, pH 4.8, containing 2 M urea. Adsorbed proteins were eluted with a linear gradient of 0–600 mM NaCl over a total volume of 240 ml at a flow rate of 60 ml/h. The inset shows the SDS–PAGE pattern (5% gel) of the fractions indicated by numbers, along with the molecular weight standard (lane M). Letters G and E show the beginning and ending breakpoints of a linear gradient, respectively. Letters T and F are the same as in Fig. 1.

it may contribute to skate cartilage developing a hard texture on the Hongtak. Similarly, it has been reported that fish with tender raw meat texture contained lower collagen contents than those with firmer textures (Hatae, Tobimatsu, Takeyama, & Matsumoto, 1986; Sato, Yoshinaka, Sato, & Shimizu, 1986; Touhata, Tanaka, Toyohara, Tanaka, & Sakaguchi, 2000).

The pepsin-solubilized collagen preparation was fractionated into three fractions, P-2.2, S-2.2 and S-11, by differential salt precipitation. The SDS–PAGE patterns of these fractions are shown in Fig. 1. The P-2.2 fraction contained two bands of alpha component, and two bands of beta component (lane 3). The S-2.2 fraction contained one band of alpha component, and one band of beta component (lane 4). Both P-2.2 and S-2.2 fractions showed quite similar SDS–PAGE patterns to Type I and II collagens, respectively, from human cartilage (Burgeson & Hollister, 1979), lamprey *Petromyzon marinus* (Kelly, Tanaka, Hardt, Eikenberry, & Brodsky, 1988), and vertebral cartilage of shark *Carcharius acutus*

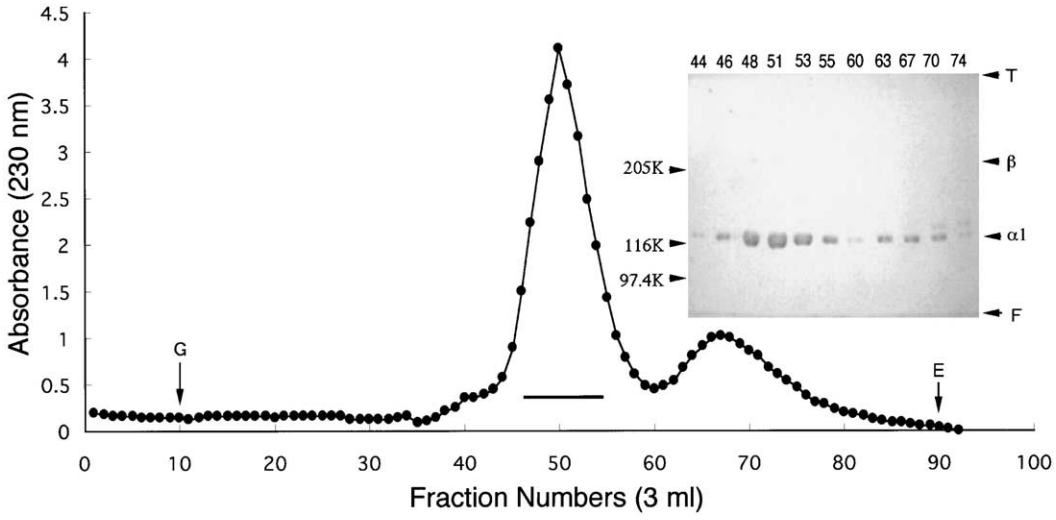

Fig. 3. Phosphocellulose column chromatography of the S-2.2 fraction. The column was equilibrated and adsorbed proteins were eluted as for the P-2.2 fraction. The inset shows the SDS–PAGE pattern (5% gel) of the fractions indicated by numbers. Letters G, E, T and F are the same as in Fig. 2.

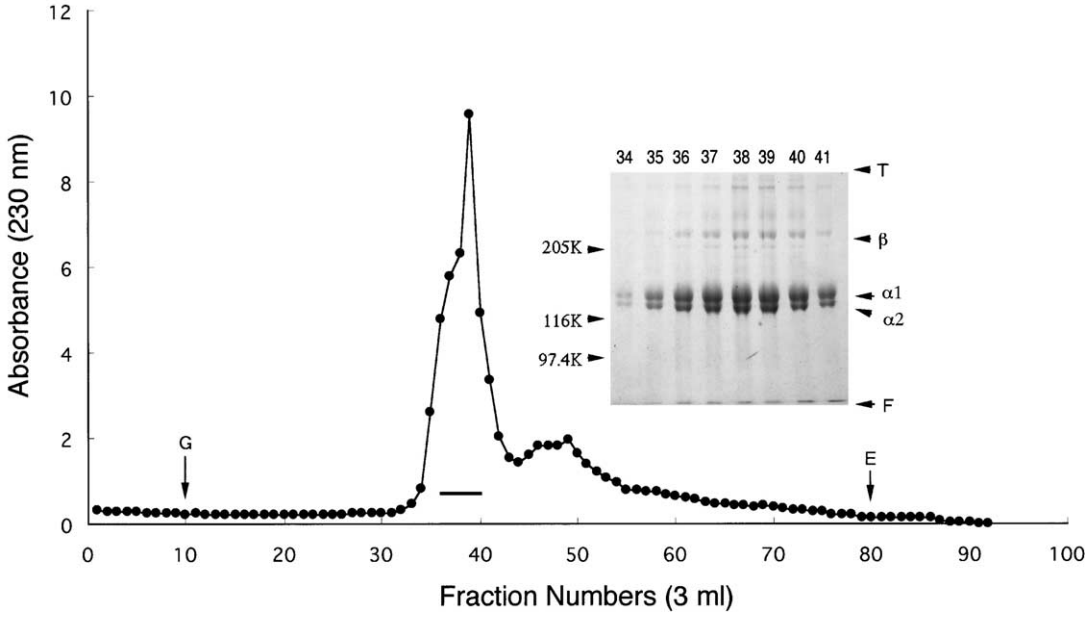

Fig. 4. Phosphocellulose column chromatography of the S-11 fraction. The column was equilibrated with 50mM sodium phosphate, pH 6.8, containing 2 M urea. Adsorbed proteins were eluted with a linear gradient of 0–800 mM NaCl over a total volume of 240 ml at a flow rate of 60 ml/h. The inset shows the SDS–PAGE pattern (5% gel) of the fractions from No. 34 to 41. Letter G and E show the beginning and end breakpoint of a linear gradient 0–800 mM NaCl, respectively. Letters G, E, T and F are the same as in Fig. 2.

(Rama Chandrakasan, 1984). On the other hand, the S-11 fraction (lane 5) contained two main bands of the alpha component with some faint bands, and showed a similar pattern to those of Type XI collagens from bovine cartilage (Ricard-Blum, Hartmann, & Ville, 1990), and notochord of lamprey *Petromyzon marinus* (Kelly et al., 1988).

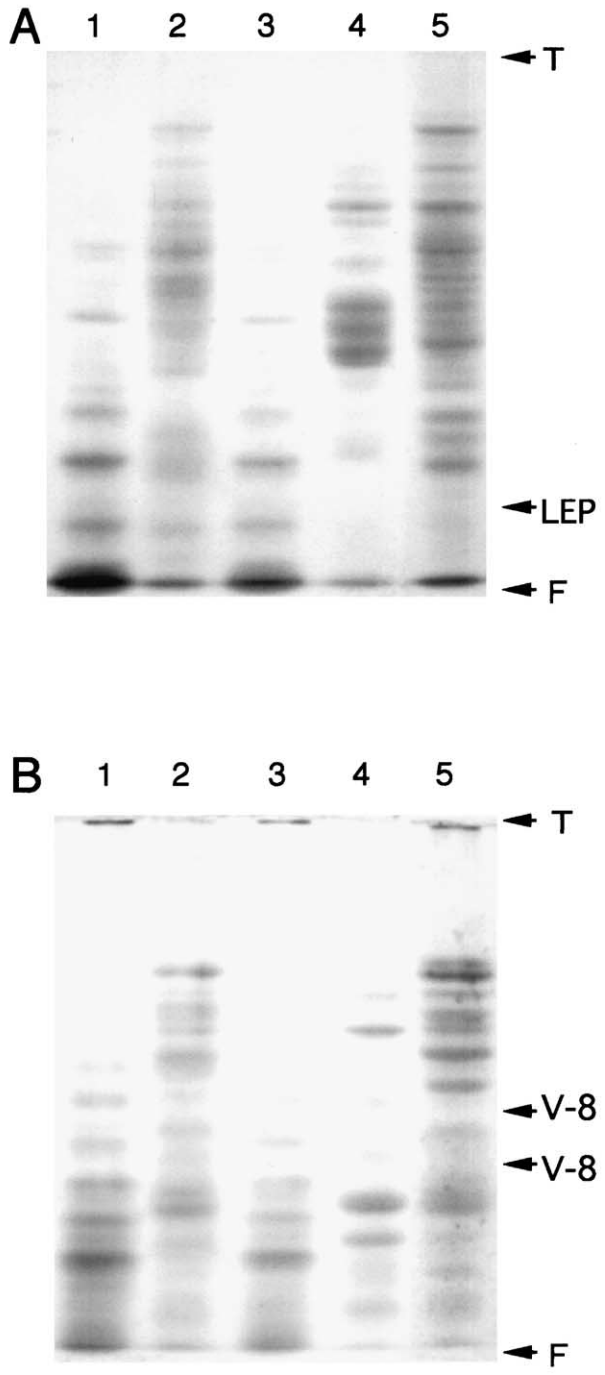

Fig. 5. Peptide maps of lysyl endopeptidase (A, 10% gel) and V-8 protease (B, 12.5% gel) digests of P-2.2 (3), S-2.2 (4) and S-11 (5) collagens from the pectoral fin cartilage of skate compared with Type I (1) and Type V (2) collagens from skate wing muscle (Mizuta et al., 2002). The arrows, LEP and V-8, show the positions of lysyl endopeptidase and V-8 protease, respectively. Letters T and F are the same as in Fig. 1.

3.2. Major collagens

Fraction P-2.2 was further purified by phosphocellulose column chromatography (Fig. 2). The P-2.2 fraction was absorbed to the column and eluted as two main peaks; however, no difference was found between former and latter peaks in the SDS–PAGE pattern (Fig. 2, inset). The underlined fractions were pooled and designated as P-2.2 collagen, and the SDS–PAGE pattern was quite similar to that of the original sample prior to chromatography (Fig. 1) and thus to the Type I collagen, which comprises two alpha bands, $\alpha 1$ and $\alpha 2$, and two bands of beta component, in skate wing muscle (Mizuta et al., 2002). Furthermore, the P-2.2 collagen showed quite similar peptide maps and amino acid compositions, especially in the higher level of alanine, to the Type I collagen in the wing muscle (Fig. 5A and 5B, lanes 1 and 3; Table 1).

Fraction S-2.2 eluted as two peaks on phosphocellulose column chromatography, one main peak with a minor peak (Fig. 3). The main peak showed similar SDS-PAGE pattern to Type II collagen (Fig. 3, inset), which is known as a major collagenous component consisting of three identical $\alpha 1$(II) chains in higher vertebrate cartilage (Miller, 1971, 1974, 1976). The underlined fractions were pooled and designated as S-2.2 collagen. The amino acid composition of S-2.2 collagen showed the distinctive features expected for Type II collagen with higher proportions of hydroxylysine and glutamic acid, and a lower proportion of alanine than P-2.2 collagen, showing clearly identifiable features in other reports (Kimura & Kamimura, 1982; Miller, 1976; Sheren et al., 1986). On the other hand, the minor peak showed a similar SDS–PAGE pattern to the main peak; however, details of the collagen type in this peak remain to be solved (Fig. 3, inset).

These combined results suggest that P-2.2 and S-2.2 collagens were genetically and structurally different from each other and should be identified as Type I and II, respectively.

3.3. Minor collagen

Fraction S-11 was further purified by phosphocellulose column chromatography, eluting as one main peak with a minor peak (Fig. 4). The underlined fractions were pooled and designated as S-11 collagen which showed almost the same solubility as that of the minor collagen (Type V) in wing muscle, however, it migrated slightly more slowly than Type V collagen (Mizuta et al., 2002). Similarly, it has reported that Type V and XI collagens functioned in the extracellular matrix not as homopolymers in their own right but copolymerized

within the fibrils that form the bulk fibrillar network, so that Type V and XI collagens can probably be classified in a same family of collagen, consisting of six different chains, α1(V), α2(V), α3(V), α1(XI), α2(XI), and α3(XI). However, the α3(XI) chain is structurally similar to the α1(II) chain (Linsenmayer, Fitch, Gross, & Mayne, 1985; Mayne, Brewton, Mayne, & Baker, 1993; Mayne & Burgeson, 1987a, 1987b; Prockop & Kivirikko, 1995; Ricard-Blum, Hartmann, Herbage, Payen-Meyran, & Ville, 1982; Reese & Mayne, 1981). On SDS–PAGE (Fig. 4, inset), S-11 collagen was quite similar to bovine Type XI collagen which appeared as a doublet of two closely spaced bands, α1(XI) and α2(XI), and was first found by Ricard-Blum et al. (1990). The amino acid composition of S-11 collagen contained a lower level of alanine and a higher level of hydroxylysine than P-2.2 and S-2.2 collagens. The feature was similar to the Type V collagen of wing muscle, except for lower levels of hydroxyproline and serine, and a higher level of methionine. These results suggest another possibility that the S-11 collagen may have lower thermal stability owing to hydrogen bonding with hydroxyproline participating in the development of thermal stability (Arai, 1991; Harrington, 1964; Zhu & Kimura, 1991). Furthermore, the S-11 collagen showed quite different peptide maps from muscle Type V collagen (Fig. 5A and 5B, lanes 2 and 5).

These combined results suggest the distribution of Type XI collagen in pectoral fin cartilage as a minor collagen.

4. Conclusion

In the previous study, we characterized the molecular species of collagen in skate wing muscle, and elucidated the distribution of Type I and V collagens (Mizuta et al., 2002). The present results suggest that three collagen types, Type I, II and XI, are genetically and structurally different molecular species of collagen, which correspond to Type I and II as major collagens and Type XI as a minor collagen in skate pectoral cartilage, respectively, based on their solubility properties, electrophoretic mobility, peptide maps, and amino acid composition. The results also suggest that collagen types in the pectoral fin may have different physiological characteristics. Further studies are in progress to elucidate subunit composition and localization of minor collagens in the skate.

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