Subunit Composition of Eel (*Anguilla japonica*) Type V Collagen: Evidence for Existence of a Novel Fourth α4(V) Chain

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Eel crude type V collagen, which was prepared by salt fractionation, was further fractionated into two fractions (A and B) by SP-Toyopearl 650M column chromatography. The subunit chains (α chains) in both fractions were isolated and characterized. Fraction A contained two distinct α chains corresponding to mammalian α1(V) and α2(V) chains (2:1). In fraction B, a novel subunit chain referred to as α4(V) chain, of which mobility on SDS–PAGE was identical to eel α2(V) chain mobility, was contained with α1(V) and α3(V) chains. Peptide mapping and amino acid analyses indicated that α4(V) chain differs from α2(V) and other known collagen chains in primary structure. Stoichiometric data on subunit composition indicate the presence of two molecular assemblies of type V collagen designated $[\alpha1(V)]_2\alpha2(V)$ and α1(V)α3(V)α4(V) in eel muscle.

INTRODUCTION

The collagen in fish muscle has been demonstrated to be closely related to the firmness of raw fish meat such as sashimi (Sato et al., 1986; Hatae et al., 1986). Collagen forms a family of connective tissue structural proteins. Until now, at least, type I and V collagens have been identified in fish intramuscular connective tissue (Sato et al., 1988, 1989a,b, 1991). Recently we found type V collagen in trout muscle softened during chilled storage to be solubilized preferentially (Sato et al., 1991). These facts indicate that the degradation of the telopeptides or cleavage of intermolecular cross-links of type V collagen plays an important role in softening of fish meat during chilled storage. However, little is known about type V collagen molecular properties, while fish muscle type I collagen has been characterized (Kimura et al., 1988; Montero et al., 1990).

The object of the present study was to ascertain the subunit composition of fish type V collagen for further study on post-mortem changes of this protein, which play an important role in postharvest textural change of fish flesh. We describe here the existence of a novel fourth subunit chain referred to as α4(V), the electrophoretic mobility of which cannot be distinguished from that of α2(V), and two molecular assemblies designated $[\alpha1(V)]_2\alpha2(V)$ and α1(V)α3(V)α4(V) in eel type V collagen.

MATERIALS AND METHODS

Materials. Live Japanese eel (*Anguilla japonica*) was used. *Staphylococcus aureus* V8 protease [EC 3.4.21.19] was purchased from Wako Chemicals (Osaka, Japan). Porcine pepsin [EC 3.4.23.1] and human placenta type V collagen were purchased from Sigma Chemical (St. Louis, MO). SP-Toyopearl 650M, a strong cation-exchange resin, was purchased from Tosoh (Tokyo, Japan); it can also be obtained from Supelco (Bellefonte, PA).

Preparation of Eel Type V Collagen. Crude type V collagen was prepared from pepsin digest of eel intramuscular connective tissue by salt fractionation according to the method of Niyibizi et al. (1984) with a slight modification (Sato et al., 1991). Crude type V collagen was dissolved in the 0.04 M Tris-HCl buffer, pH 8.2, containing 2 M urea and 0.05 M NaCl (50 mg/150 mL) and loaded onto a column (10 cm × 2.5 cm i.d.) packed with SP-Toyopearl 650M equilibrated with the same buffer by a Perista pump (ATTO, Tokyo, Japan) at 230 mL/h. Elution was performed by using a linear gradient from 0.1 to 0.4 M NaCl in the sample buffer in a total volume of 400 mL. The fractions containing type V collagen molecules were pooled and dialyzed against 5 mM acetic acid and lyophilized.

Electrophoresis. Sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) was performed according to the method of Laemmli (1970). The gel was developed by using Coomassie Brilliant Blue R-250. In some cases, the density of each component was estimated by scanning the gel at 525 nm with a Chromato-Scanner CS 930 (Shimadzu, Kyoto, Japan).

For recovery of protein from the gel, a glass tube (50 mm × 5 mm i.d.) was prepared. Three hundred microliters of 3% stacking gel solution (Laemmli, 1970) was pipetted into the tube and then polymerized. After polymerization of the stacking gel, the tube was filled with 125 mM Tris-HCl buffer, pH 6.8, containing 0.1% SDS and 1 mM ethylenediaminetetraacetic acid. The band of interest was visualized with Coommassie Brilliant Blue R-250 according to the method of Cleaveland et al. (1977), cut from the preparative gel, and put into the tube. Then the tube was attached to a disc gel electrophoresis apparatus (ATTO SJ-1060, Tokyo, Japan), the top and bottom chambers of which were filled with the electrode buffer of Laemmli (1970). The protein in the preparative gel was electrophoretically eluted (10 mA/tube for 2 h) into a microdialysis cup (ATTO Desalyzer I, AE-6590, Tokyo, Japan).

Peptide Mapping. The proteins electrophoretically eluted from the preparative gel were digested with *S. aureus* V8 protease according to by the method of Cleaveland et al. (1977) at an enzyme/substrate ratio of 1:20. Peptides generated by the protease digestion were analyzed by SDS–PAGE.

Separation of α Chains by High-Performance Liquid Chromatography (HPLC). Purified type V collagen molecules were resolved into subunits in 10 mM potassium phosphate buffer, pH 6.00, at 50 °C (1 mg/mL) and clarified by centrifuge before injection to a Bakerbond WP CBX scout column (J. T. Baker, Phillipsburg, NJ). Bound protein was eluted at 1 mL/min by increasing the concentration of potassium phosphate buffer. Each α chain eluted from the column was collected and applied to a Bakerbond WP butyl scout column. Elution was performed by a linear gradient from 10 to 50% acetonitrile in the presence of 0.1% (v/v) TFA over 30 min at 1 mL/min.

Amino Acid Analysis. Hydrolysis of protein and amino acid analysis were performed according to the method of Bidlingmeyer et al. (1984) with a slight modification (Sato et al., 1992).

RESULTS

Purification of Type V Collagen Molecules. SDS–PAGE patterns of eel crude type V collagen prepared by conventional salt fractionation showed three distinct α

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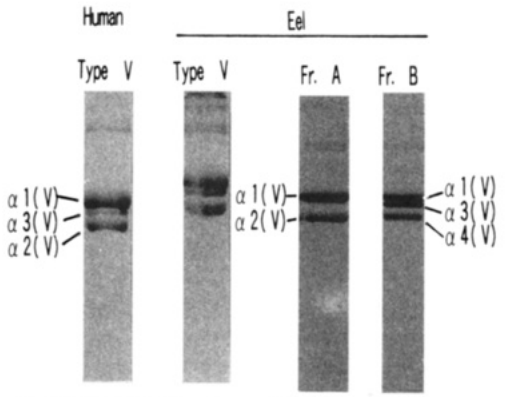

Figure 1. SDS–PAGE patterns (7.5% gel) of human and eel type V collagens. Eel crude type V collagen was fractionated into fractions A and B by SP-Toyopearl 650M column chromatography as shown in Figure 2.

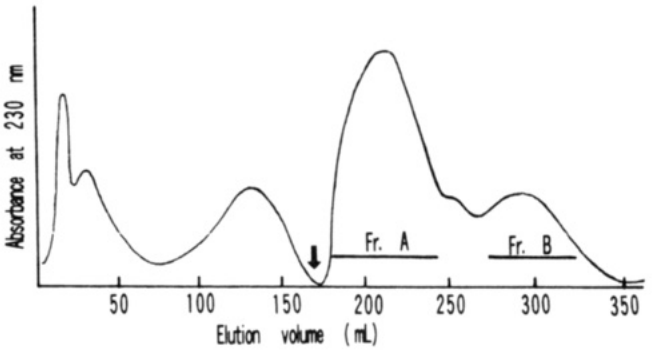

Figure 2. Fractionation of eel crude type V collagen by SP-Toyopearl 650M column chromatography. Fractions marked A and B were collected. Arrow shows the start of the gradient elution. For experimental details, see Materials and Methods.

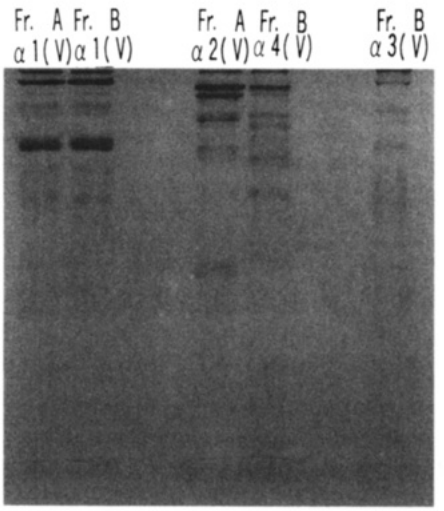

Figure 3. Peptide mapping of eel type V collagen subunits. α1(V), α2(V), α3(V), and α4(V) chains in fractions A and B as shown in Figure 1 were recovered from the gel and digested with *S. aureus* V8 protease at 37 °C for 30 min and analyzed on 12.5% gel.

chains with electrophoretic mobilities corresponding to human α1(V), α2(V), and α3(V) chains (Figure 1). Crude type V collagen was further fractionated by SP-Toyopearl 650 M column chromatography. As shown in Figure 2, two major peaks were eluted in the NaCl gradient (fractions A and B). SDS–PAGE analysis showed that fraction A contained two distinct α chains with electrophoretic mobilities corresponding to human α1(V) and α2(V) chains in the ratio of approximately 2:1, which will be referred to as eel α1(V) and α2(V) chains, respectively (Figure 1).

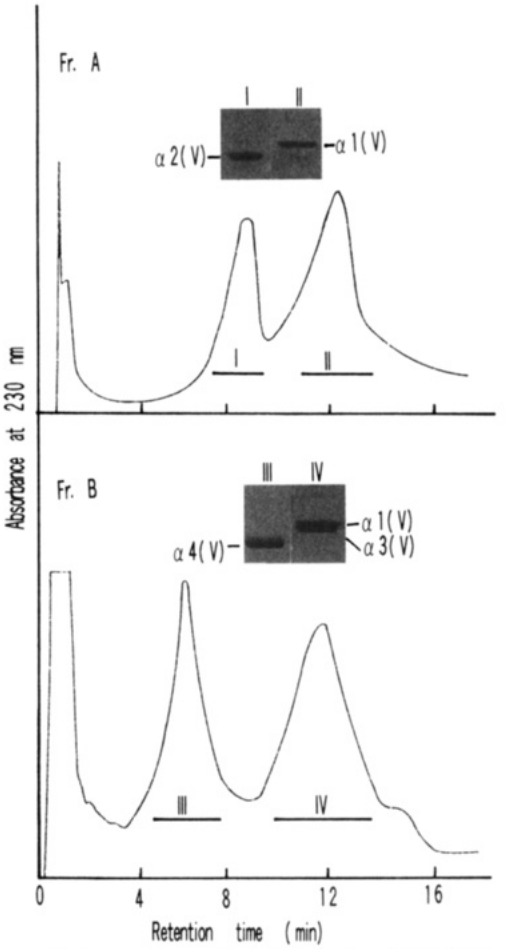

Figure 4. Cation-exchange HPLC (Bakerbond WP CBX) elution profiles for type V collagen subunits (fractions A and B). Elution was performed with a linear gradient from 10 to 155 mM potassium phosphate buffer, pH 6.0 at 1 mL/min over 16 min. (Insets) SDS–PAGE patterns of the pooled fractions indicated with bars.

On the other hand, fraction B contained three distinct α chains with electrophoretic mobilities corresponding to human α1(V), α2(V), and α3(V) chains in the ratio 1:1:1 (Figure 1). The following peptide mapping and amino acid analyses will reveal that the band in fraction B with the electrophoretic mobility corresponding to eel α2(V) chain is different in primary structure from α2(V) chain. Then in the discussion which follows, the three α chains in fraction B will be referred to as eel α1(V), α3(V), and α4(V) chains as illustrated in Figure 1. The unadsorbed effluent from the column contained a small amount of collagen which had disulfide bonds in its molecule (data not shown).

Peptide Mapping. As shown in Figure 3, the α1(V) chain in fraction A showed the same peptide pattern as the corresponding α chain in fraction B, indicating that the primary structures of both chains are identical. On the other hand, the peptide pattern of the α2(V) chain in fraction A was different from that of the α4(V) chain in fraction B, whereas both chains cannot be distinguished by SDS–PAGE. Shortened and prolonged incubation times with the protease yielded the same results. Eel α3-(V) chain showed a peptide pattern different from that of the others.

Separation of Type V Collagen α Chains by HPLC. Eel type V collagen subunits in fractions A and B were separated by cation-exchange HPLC (Bakerbond WP CBX). As shown in Figure 4, α1(V) and α2(V) chains in

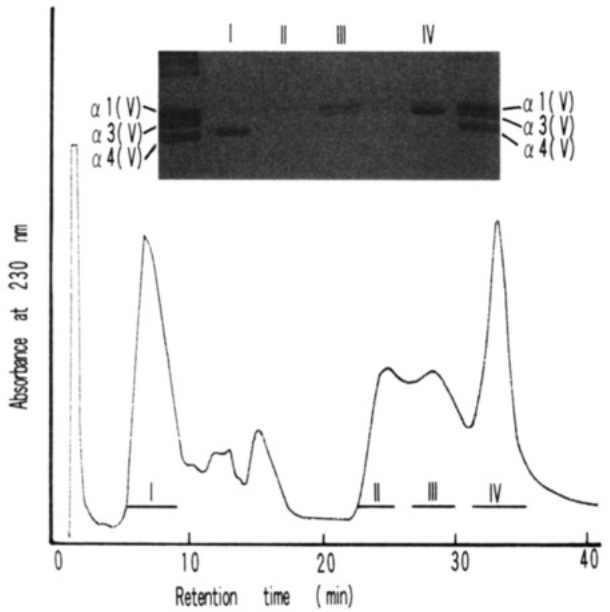

Figure 5. Cation-exchange HPLC (Bakerbond WP CBX) separation of α3(V) chain by combination of isocratic and gradient elutions. Solvent A consists of 10 mM potassium phosphate buffer, pH 6.0, solvent B of 100 mM potassium phosphate buffer, pH 7.0. Initial, 0% B; from injection to 8 min, 20% B; 8–10 min, 20–40% B; 10–18 min, 40% B; 18–20 min, 40–60% B; 20–28 min, 60% B; 28–30 min, 60–80% B; 30–38 min, 80% B; 38–40 min, 80–100% B. (Inset) SDS–PAGE patterns of pooled fractions indicated by bars.

fraction A could be separated by a linear gradient of phosphate buffer. In fraction B, the α4(V) chain could be separated from α1(V) and α3(V) chains, while the α1(V) chain was coeluted with the α3(V) chain. It should be noted that the retention time of α2(V) in fraction A was different from that of α4(V) in fraction B. Separation of the α3(V) chain from the α1(V) chain was achieved by a multistep linear gradient elution. As shown in Figure 5, four peaks appeared by this elution condition; the α3(V) chain was recovered in the fourth peak. Isolated α chains were applied to a reversed-phase HPLC (Bakerbond WP butyl). In all cases, only a single protein peak appeared (data not shown). Protein peaks were collected and then lyophilized.

Amino Acid Composition of α Chain. The amino acid compositions of the HPLC-purified eel type V collagen subunits are shown in Table 1 along with those of human α1(V), α2(V), and α3(V) chains (Sage and Bornstein, 1979). Eel α1(V), α2(V), and α3(V) chains showed typical compositional features of the corresponding human chains except for a slightly higher content of proline and lower contents of aspartic acid, valine, isoleucine, leucine, and hydroxylsine. On the other hand, the eel α4(V) chain was characterized by significantly higher contents of glycine and alanine and lower contents of histidine and valine than eel and human α2(V) chains. We believe that the α4(V)-like chain has not been isolated from mammalian and fish tissues until now.

DISCUSSION

It has been demonstrated that type V collagen in mammalian tissues consists of three distinct subunits designated α1(V), α2(V), and α3(V) chains (Burgeson et al., 1976; Chung et al., 1976; Brown et al., 1978). In addition, Fessler et al. (1985) identified a fourth α chain in chicken tissue. As the helical region of the fourth chicken α chain could not be distinguished from the α1-(V) chain, it has been referred to as the α′1(V) chain (Fessler et al., 1985). The present study shows that eel intramuscular type V collagen molecules consist of four distinct α chains. Three of them correspond to mammalian α1(V), α2(V), and α3(V), respectively, while the fourth eel α chain differs in amino acid composition of the helical region from other type V collagen chains and any other known α chains from fish collagen (Kimura and Ohno, 1987; Ramshaw et al., 1988). Thus, we propose to name it eel α4(V) chain.

The native eel type V collagen prepared by salt fractionation can be fractionated chromatographically into two fractions (see Figure 2). Type V collagen molecules in both fractions contained α1(V) chain with α2(V) chain (fraction A) or with α3(V) and α4(V) chains (fraction B). On the other hand, eel α1(V) chains from the both fractions have the same retention time on cation-exchange HPLC after resolving into subunits (see Figure 4). These facts indicate that α1(V) chain assembles in molecules with α2-(V) chain in a molecular ratio of 2:1 (fraction A) or with α3(V) and α4(V) chains in a ratio of 1:1:1 (fraction B). There is a possibility that other homotrimer and/or heterotrimer forms exist in eel type V collagen. However, it has been demonstrated that the ratio of the sum of fish α1(V) and α3(V) chains to the sum of α2(V) and α4(V) chains is almost constant, whereas the ratio of α1(V) to α3(V) chains differs among preparations (Sato et al.,

Table 1. Amino Acid Composition of Eel α1(V), α2(V), α3(V), and α4(V) Chains and of Human Type V Collagen Subunits

	eel type V collagen[a]				human[b]		
	α1(V)	α2(V)	α3(V)	α4(V)	α1(V)	α2(V)	α3(V)
Asp	40.8 ± 2.3	35.2 ± 1.2	42.9 ± 0.2	36.1 ± 2.7	50	50	42
Glu	101.0 ± 6.1	94.8 ± 1.8	99.3 ± 0.7	84.0 ± 1.1	100	88	98
Hyp	98.5 ± 4.2	100.4 ± 2.0	82.4 ± 3.5	97.0 ± 0.7	100	108	91
Ser	39.0 ± 1.3	45.0 ± 1.2	38.9 ± 2.6	39.1 ± 1.5	23	34	34
Gly	325.5 ± 4.7	338.5 ± 5.8	336.0 ± 3.1	353.8 ± 2.2	325	325	332
His	7.9 ± 0.3	8.2 ± 0.3	7.0 ± 0.3	2.8 ± 0.1	6	10	14
Arg	46.3 ± 0.6	56.5 ± 1.2	45.0 ± 1.0	54.3 ± 0.5	42	52	42
Thr	27.4 ± 0.8	33.9 ± 0.7	21.3 ± 1.7	23.4 ± 0.2	22	26	19
Ala	42.4 ± 2.1	53.7 ± 1.1	50.8 ± 0.5	86.2 ± 1.7	41	57	49
Pro	147.9 ± 2.7	123.3 ± 5.7	137.7 ± 3.8	132.2 ± 1.3	125	105	99
Tyr	3.5 ± 0.4	1.2 ± 0.3	3.0 ± 1.1	1.1 ± 0.0	3	2	2
Val	15.1 ± 1.3	23.3 ± 1.3	16.7 ± 0.6	11.0 ± 0.6	21	31	29
Met	6.7 ± 0.6	8.7 ± 0.3	7.9 ± 0.9	5.9 ± 0.7	7	10	8
Ile	11.2 ± 1.0	9.3 ± 0.4	16.0 ± 0.5	9.2 ± 0.2	20	18	20
Leu	32.8 ± 1.7	28.7 ± 1.3	36.7 ± 1.9	22.6 ± 1.2	44	39	56
Hyl	28.7 ± 1.4	14.4 ± 0.6	32.6 ± 1.0	16.1 ± 1.1	46	23	43
Phe	11.2 ± 0.6	9.9 ± 0.3	11.4 ± 0.8	12.6 ± 0.5	12	11	9
Lys	14.1 ± 0.9	14.8 ± 0.3	14.8 ± 1.0	12.4 ± 0.2	15	13	15

[a] Each analysis represents the mean ± standard deviation of three determinations. [b] Sage and Bornstein (1979).

1989b). It is unlikely, then, that a significant amount of, if any, homotrimer forms of type V collagen is present in eel intramuscular connective tissue. In the previous paper (Sato et al., 1989b), we assumed that fish type V collagen had two molecular forms designated $[\alpha1(V)]_2\alpha2(V)$ and $\alpha1(V)\alpha2(V)\alpha3(V)$ on the basis of electrophoretic patterns. The present study, however, demonstrates that the $\alpha2(V)$ chain is not contained in fraction B, which contains $\alpha3(V)$ chain (see Figure 4). The same results were obtained in other eel specimens, indicating that no significant amount of the $\alpha1(V)\alpha2(V)\alpha3(V)$ molecule is contained in eel intramuscular connective tissues. On the basis of stoichiometric data on subunit compositions of eel type V collagen molecules, we conclude that the major molecular forms of type V collagen in eel intramuscular connective tissue are $[\alpha1(V)]_2\alpha2(V)$ and $\alpha1(V)\alpha3(V)\alpha4(V)$.

The presence of $\alpha4(V)$ chain in carp (*Cyprinus carpio*) and rainbow trout (*Oncorhynchus mykiss*) has been confirmed by using the cation-exchange HPLC separation (data not shown). Thus, the $\alpha4(V)$ chain is widely distributed in fish type V collagen. On the other hand, the presence of fish $\alpha4(V)$-like chain has not been described in avian and mammalian type V collagens [see the recent review by van der Rest and Garrone (1991)]. There is a possibility that fish $\alpha4(V)$ chain has disappeared or changed into other collagen chains during evolution. However, there is another possibility that fish $\alpha4(V)$-like chain and $\alpha1(V)\alpha3(V)\alpha4(V)$ molecule might be present in higher vertebrates. Whereas fish $\alpha4(V)$ chain cannot be distinguished from $\alpha2(V)$ chain by SDS–PAGE, subunits of mammalian type V collagen molecule have been examined by only SDS–PAGE (Rhodes and Miller, 1981; Niyibizi et al., 1984; Schuppan et al., 1986; Hashimoto et al., 1988). To resolve these hypotheses, re-examination of vertebrate type V collagen subunit composition is required.

The present study revealed the presence of two distinct type V collagen molecular forms and their four subunits in eel muscle. On the basis of the present results, further studies on post-mortem changes of fish type V collagen in association with softening of fish meat during chilled storage are now in progress.

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