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Characterization of molecular species of collagen in scallop mantle

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Abstract

Pepsin-solubilized collagen (SMPC) extracted from scallop mantle was first subjected to amino acid analysis and molecular weight determination. The SMPC was rich in hydroxylysine. After salting out, the SMPC showed two different profiles in molecules, amino acids and peptide maps. The two different collagen molecules had different uronic acid contents and gel-forming abilities. SMPC may be applicable to a variety of usage, including functional foods.

Keywords: Collagen; Scallop mantle; Mollusk; Molecular species

1. Introduction

Approximately 30% of proteins in animal tissues are collagens and play important roles as tissue matrices. In the fields of food science and industry, the amount and properties of the composed collagen in meat have become an important index for texture evaluation. Mizuta, Yamada, Miyagi, and Yoshinaka (1999), Olaechea, Ushio, Watabe, Takeda, and Hatae (1993), Sato, Yoshinaka, Sato, and Shimizu (1986) reported a relationship between the texture and the collagen properties of marine vertebrates. Kim, Byun, Park, and Shahidi (2001), Kim et al. (2001) and Sato et al. (1986) reported some features and functionalities of marine vertebrate collagen peptides. In contrast to the wealth of studies on vertebrate collagens, including those from marine sources, studies on collagen of invertebrates which occupies 95% of the whole animal kingdom have been very limited (Elijah, 1978). In this study, we analyzed collagen molecular species of scallop mantle, since scallop mantle is consistently by-produced (30,000 tons per year in Hokkaido island, Japan), and it may become a sustainable source of useful collagens for various purposes, including value-added foods.

2. Materials and methods

2.1. Materials

Frozen scallop (*Patinopecten yessoensis*) mantles were obtained from Hokkaido Monbetsu fisheries experimental station, located in Monbetsu city, Japan.

2.2. Preparation of scallop mantle collagen

All operations were done below 4 °C. Scallop mantles were homogenized with 5 volumes (v/w) of distilled water, using a homogenizer. The homogenate was then centrifuged at 10,000*g* for 20 min. To the precipitate, 20 volumes (v/w) of 0.1 N NaOH were added. The suspension was stirred overnight and then centrifuged at 10,000*g* for 20 min. This alkaline extraction was thrice repeated. The precipitate was washed thoroughly with distilled water. Then the precipitate was suspended in 10 volumes (v/w) of 0.5 M acetic acid and solubilized by limited digestion with porcine pepsin (Sigma chemical) at a collagen:pepsin weight ratio of 100:5. The digestion was done again for another 2 days. The resulting viscous solution was centrifuged at 10,000*g*

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for 1 h, and supernatant thus obtained was adjusted to 0.7 M NaCl by adding 4.0 M NaCl to precipitate the solubilized collagen. The resulting precipitate was collected with low speed centrifugation, redissolved in 0.5 M acetic acid, and then centrifuged at 30,000*g* for 1 h. Finally, the supernatant was dialyzed against distilled water and lyophilized.

2.3. Fractionation of collagen

Two hundred milligram of SMPC was dissolved in 100 ml of 0.5 M acetic acid, or 100 ml of 1.0 M NaCl containing 0.05 M Tris–HCl buffer (pH 7.5). The final NaCl concentrations were adjusted to 0.2 M, 0.3 M, and 0.45 M for acetic acid-soluble collagen, and 2.8 M, 3.5 M, and 4.0 M, for Tris–HCl buffer-soluble collagen, respectively. The precipitates then appearing were collected by centrifuging (10,000*g*, 30 min). All precipitated fractions were again dissolved in 0.5 M acetic acid, and dialyzed against distilled water to remove salts.

2.4. Amino acid composition

Collagen samples were hydrolyzed in 6 M HCl at 110 °C for 24 h, and the hydrolysates were analyzed by the amino acid analyzer JLC-500N (Electron Japan).

2.5. Electrophoresis

Sodium dodecylsulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was performed according to the method of Laemmli (1970). Proteins were stained with Coomassie brilliant blue R-250, then destained with 5% methanol and 7.5% acetic acid.

2.6. Cyanogen bromide digestion

The two collagen species purified from the scallop mantle were lyophilized and reconstituted in 70% formic acid solution at 2 mg/ml. Cyanogen bromide crystals (20 mg/ml) were added to the collagen solution. Nitrogen gas was bubbled through the mixture and the digestive reaction was carried out in sealed tubes at 25 °C for 18 h. The mixtures were centrifuged and the supernatant was dialyzed against 0.01 M Tris–HCl (pH 7.4) to remove excess cyanogen bromide. The obtained digest was loaded onto a 10% SDS-PAGE, and stained with Coomassie blue.

2.7. V8 protease digestion and peptide mapping

Samples, of 200 μg each, were dissolved in 0.5 M phosphate buffer (pH 7.2), containing 0.5% SDS, and boiled for 1 min. After adding 10 μl of the same buffer, 5 μg of V8 protease were added to each of the collagen solutions, then incubated at 37 °C for 25 min. Digestion was quenched by boiling for 3 min and subjected to electrophoresis directly with 10% SDS polyacrylamide gels.

2.8. Gel-forming capacity

Gel-forming capacity of the scallop mantle collagen was evaluated as follows: to 1.5 ml of Na-phosphate buffer of specific pH, 1.5 ml of the 0.3% (w/v) collagen diluted in HCl solution (pH 3) were added. After immediate mixing, the solution was kept below 4 °C for 30 min. The resulting gel formation was monitored by measuring the absorbance at 310 nm as turbidity change. The pHs were fixed to 5.5, 6.0, 6.5, 6.8, 7.0, 7.5 and 7.9 with buffers. The buffer contained NaCl to make the final concentrations in the gels 0, 20, 50, 100, and 150 mM.

2.9. Uronic acid analysis

Uronic acid was determined by the sulfuric acid-carbazole method (Bitter & Muir, 1964). A calibration curve was prepared using glucuronic acid as a standard.

3. Results and discussion

3.1. Extraction of the collagen and its purity

Through limited pepsin degradation, 479 mg of lyophilized SMPC were obtained from 100 g scallop mantle. After heating the SMPC for 3 min, it was subjected to SDS-PAGE. As shown in Fig. 1, two α-chains with molecular weights of around 150 kDa and 130 kDa, and a β-chain, above those α-chain bands, were observed. There was no prominent band other than those three. The molecular weight of the two α-chains coincided well with that of bovine placenta type V collagen (Shen, Ono, Kurihara, & Takahashi, 2005). There was no difference in SDS-PAGE patterns between 2-mercaptoethanol-treated SMPC and

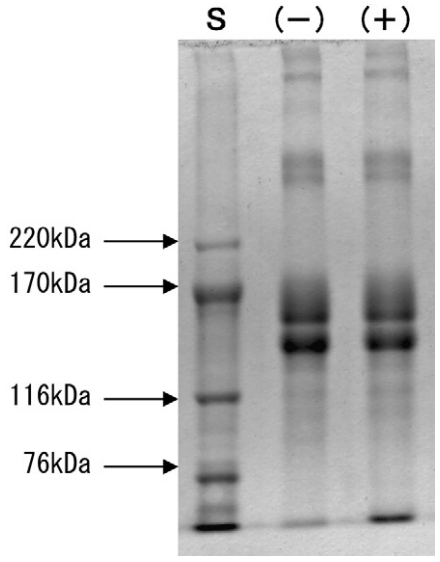

Fig. 1. SDS-PAGE (5% gel) patterns of scallop mantle collagen with (+) or without (−) 2-mercaptoethanol reduction. S: molecular weight marker.

native SMPC, implying that there are no disulfide linkages between chains. For this reason, molecular species of types III and IV collagens would not appear in the scallop mantle collagen. The obtained collagen contained glycine, reaching up to 331 residues per 1000 amino acid residues. This is a feature of the amino acid composition of collagen.

3.2. Fractionation of scallop mantle pepsin-solubilized collagen

SMPC dissolved in 0.5 M acetic acid was precipitated by salting out. Most of the collagens were precipitated at a concentration of 0.45 M NaCl. But, when collagen was dissolved by neutral buffer solution, most of them were finally precipitated at 4.5 M NaCl. Through fractionation of scallop mantle pepsin-solubilized collagen with various concentrations of NaCl in acetic acid solution, the SDS-PAGE patterns of 0.2–0.3 M NaCl precipitate (lane H) and 0.3–0.45 M NaCl precipitate (lane I) fractions differed from that of the neutral Tris–HCl buffer solution as shown in Fig. 2. The band width of α_1-chains in lanes H and I were narrower than those in the other lanes.

We compared the amino acid composition between the $P_{0.2}$ and $P_{0.45}$. And indeed there was a difference between these two, as seen in Table 1. As well as type I and type V collagens, major and minor collagens have been fractionated from various marine animal tissues (Kimura, Ohno, Miyauchi, & Uchida, 1987; Kimura, Zhu, Matsui, Shijoh, & Takamizawa, 1988; Nishimoto, Mizuta, & Yoshinaka, 2004; Sato, Yoshinaka, Sato, Itoh, & Shimizu, 1988; Sato, Yoshinaka, Itoh, & Sato, 1989). There is a report on marine invertebrate collagen which was shown to have characteristics similar to mammalian type Vcollagen, and workers have succeeded in separating those molecular species (Mizuta, Miyagi, & Yoshinaka, 2005; Sivakumar, Suguna, & Chandrakasan, 2000). However, there are no similar reports on scallop mantle. Collagen from the 0.2–0.45 M NaCl precipitated fraction had more hydroxylysine than that from the 0–0.2 M NaCl-precipitated fraction. There were some other smaller differences in amino acid composition (other than hydroxylysine). The scallop mantle minor collagen had fewer hydrophilic amino acids than did the major collagen. There were several side-chained hydrophobic amino acids of large molecular weight in the minor collagen. We speculated that the major collagen of the scallop mantle had a similar role to the mammalian type I collagen, and that the minor collagen of the scallop to the type V collagen in the tissue. However, the amino acid compositions of both major and minor collagens were comparable to those of the corresponding vertebrate collagens (Nishimoto et al., 2004; Nishimoto, Sakamoto, Mizuta, & Yoshinaka, 2005). This supports a recent speculation (Sivakumar et al., 2000; Sivakumar, Suguna, & Chandrakasan, 2003). Namely, the invertebrate collagen, which resembles vertebrate type V collagen, possibly did not evolve as did the vertebrate type I collagen.

Table 1
Amino acid compositions of scallop mantle collagen and two NaCl precipitated fractions[a]

Amino acid	Collagen	$P_{0.2}$	$P_{0.45}$
Asp	58	57	56
Thr	29	29	26
Ser	50	51	46
Glu	111	110	105
Gly	331	330	327
Ala	54	56	47
Val	19	18	21
Met	24	23	25
Ile	18	17	20
Leu	31	30	35
Tyr	6	6	6
Phe	10	10	11
HyLys	16	15	23
Lys	7	7	7
His	5	4	5
Arg	50	50	50
Hypro	84	87	89
Pro	97	100	101

$P_{0.2}$ and $P_{0.45}$ designate 0–0.2 M and 0.2–0.45 M NaCl concentrations for collagen precipitation, respectively.
[a] Residues per 1000 residues.

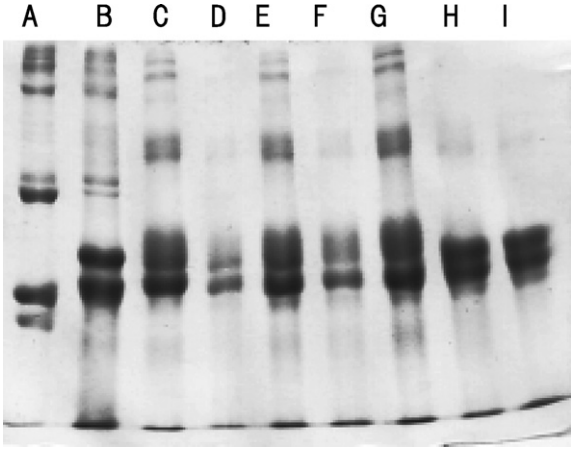

Fig. 2. SDS-PAGE (5% gel) patterns of precipitated fractions of the scallop mantle pepsin-solubilized collagen treated with varying concentrations of NaCl in 0.5 M acetic acid and 1.0 M Tris buffer (pH 7.5): A, bovine skin type I collagen; B, bovine placenta type V collagen; C, scallop mantle pepsin-solubilized collagen; D, precipitate in 2.5–2.8 M NaCl/Tris buffer; E, precipitate in 2.8–3.5 M NaCl/Tris buffer; F, precipitate in 3.5–4.0 M NaCl/Tris buffer; G, precipitate in 0.2 M NaCl/acetic acid; H, precipitate in 0.2–0.3 M NaCl/acetic acid; I, precipitate in 0.3–0.45 M NaCl/acetic acid.

3.3. Mapping of the collagen peptides

To confirm the difference in primary structure of the two isolated collagens, cyanogen bromide and V8 protease degradations were done. The resulting peptides were mapped by SDS-PAGE gel as shown in Figs. 3 and 4. The two collagens showed different mapping profiles, implying a different primary structures.

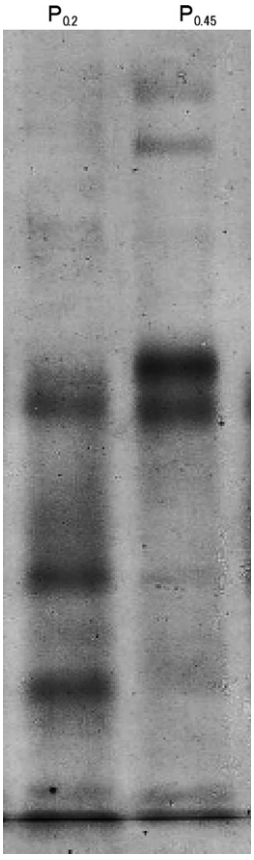

Fig. 3. Peptide maps of V8-protease digests obtained from $P_{0.2}$ and $P_{0.45}$ collagens of the scallop mantle. See Table 1 for $P_{0.2}$ and $P_{0.45}$.

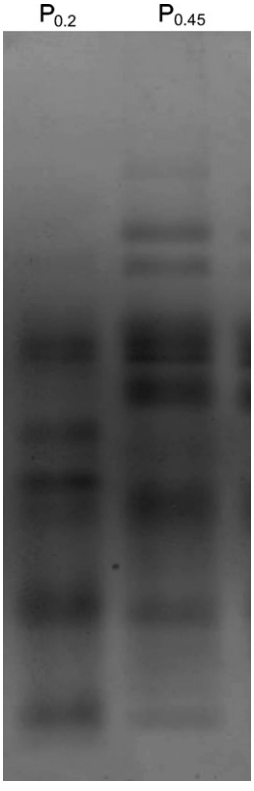

Fig. 4. Peptide maps of cyanogen bromide digests obtained from $P_{0.2}$ and $P_{0.45}$ collagens of the scallop mantle. See Table 1 for $P_{0.2}$ and $P_{0.45}$.

3.4. Gel-forming ability

SMPC was subjected to gel-formation analysis below 4 °C. Gel-forming was greatly influenced by ionic strength and pH (Fig. 5). The highest gel-forming condition was observed when 150 mM NaCl and phosphate buffer, pH 6.0, were employed. This suggests that the gel-forming styles between the scallop mantle collagen and that from vertebrate differ. For example, under the above condition, bovine type I collagen does not form gel. But once the pH is adjusted to neutral, and by raising the temperature, a turbid strong gel appeared. This implies that the habitat and tissue structure are different from mammals, resulting in the lower ability to form gels (Shen, Ono et al., 2005). By forming a gel, using major and minor collagens from scallop mantle athero collagen with 150 mM NaCl and pH 6 phosphate buffer, it became clear that a gel more easily forms with major collagen than with minor collagen (Fig. 6). The lower gel-forming ability of minor collagen might be due to the higher hydroxylysine content. Hydroxylysine is considered to bind with sugar (Kimura, 1972). For this reason, sugar may impair the forming of the gel.

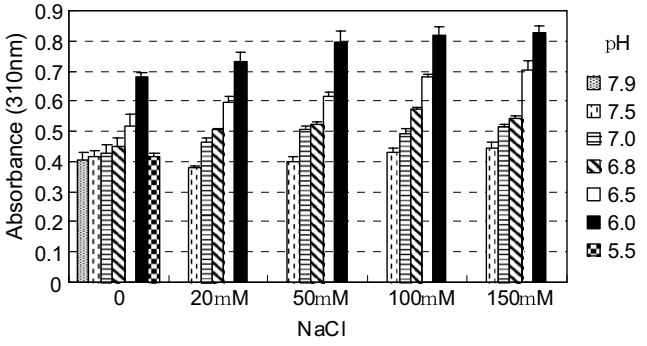

Fig. 5. Effect of pH and NaCl concentration on scallop mantle collagen gel-formation at 4 °C. Gel-formation was evaluated as increase in turbidity at 310 nm. Values are means ± standard deviation ($n = 3$).

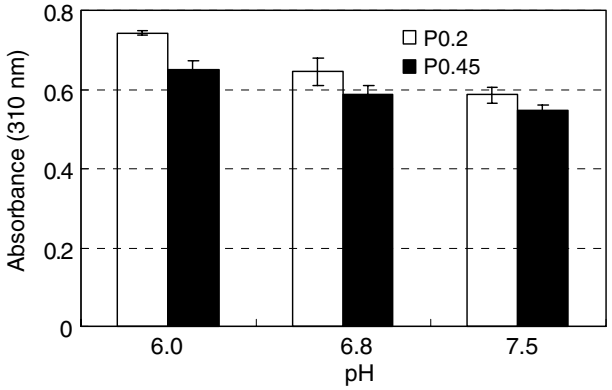

Fig. 6. Gel-formation capacities of two collagens species, $P_{0.2}$ and $P_{0.45}$. See Table 1 for $P_{0.2}$ and $P_{0.45}$.

3.5. Uronic acid content of scallop mantle collagen

The amount of uronic acid in minor collagen was twice that in major collagen. Specifically, it was $0.948 \pm 0.058\%$ for minor collagen and $0.521 \pm 0.026\%$ for major collagen. At first, we considered that the wide band of α-chain appearing in the upper portion of the SDS-PAGE indicated glucosaminoglucan. But this was ruled out by the fact that, even though minor collagen contains more uronic acid, it shows a much narrower band in SDS-PAGE. To elucidate this phenomenon, we carried out anion and gel filtration chromatographies for separating the α-chain molecular species. It was found that scallop mantle collagen consisted of three α-chains (Shen, Kurihara, & Takahashi, 2005). The reason why the upper band of the α-chain in major collagen was wide might be coexistence of two α-chains, α_1 and α_3. Since minor collagen showed a sharp band on the SDS-PAGE, three different α-chains are considered to originate from major collagen. Cation-exchange chromatography has been used to separate the α-chain of mammalian type I collagen. But cation-exchange chromatography is not applicable to type V collagen of vertebrates and also it was not applicable to the scallop mantle major collagen. On the other hand, anion-exchange chromatography was applicable to both collagens. Scallop mantle collagen and type V collagen have very similar molecular species of major collagen. For these reasons, scallop mantle major collagen may have a beneficial characteristic, comparable to the type V collagen of vertebrates. Therefore, it maybe useful in a variety of applications as an alternative to type I collagen of vertebrates which has been widely used. Scallop mantle major collagen also has a unique amino acid composition (rich in hydroxylysine), and a very low gel-forming ability in 0.45 M NaCl precipitated collagen. Scallop mantle collagen would open a new field for various purposes, including value-added functional foods.

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