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Characterization and subunit composition of collagen from the body wall of sea cucumber *Stichopus japonicus*

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Abstract

Pepsin-solubilized collagen (PSC) without telopeptides was prepared from the body wall of the sea cucumber *Stichopus japonicus* and isolated by selective precipitation with NaCl. The PSC exhibited a maximum absorbance at 220 nm. The subunit of PSC was isolated by Sephacryl S-300 HR. The results of SDS-PAGE suggested that purified collagen from *S. japonicus* was a 1α trimer (about 135 kDa) while 1α chain resembling α_1 chain of type I collagen of vertebrate. The thermal stability temperature (T_s) was 57.0 °C as measured by DSC, about 5.0 °C lower than that of type I collagen of calf. Peptide mapping and amino acid analysis of PSC also revealed the difference between invertebrate and vertebrate. However, the presence of $(\alpha_1)_3$ trimers was evident.

Keywords: Collagen; *Stichopus japonicus*; Sea cucumber; Thermal stability; Type I

1. Introduction

Sea cucumbers (*S. japonicus*) belong to the phylum *Echinodermata* and are traditional tonic consumed by Chinese and Japanese. The major edible parts of sea cucumber are the body wall mainly consisting of collagen and mucopolysaccharides. More than 21 types of collagen have so far been identified in various tissues and their roles have been investigated (Gelse, Poschl, & Aigner, 2003). Collagen peptides are believed to have a high water retention capacity, a high anti-radical activity and a high potential for decreasing blood pressure (Byun & Kim, 2001; Fahmi et al., 2004; Morimura et al., 2002; Tabata & Ikada, 1998). In invertebrate, at least two types of collagen, types I and V collagens, are found to exist (Kimura & Tanaka, 1983; Sivakumar, Suguna, & Chandrakasan, 2000). The collagen of sea urchin has been found to be with two distinct subunits, α_1 and α_2, forming $(\alpha_1)_2\alpha_2$ which resemble that of type I collagen (Cluzel, Lethias, Garrone, & Exposito, 2000; Omura, Urano, & Kimura, 1996; Robinson, 1997).

There is little information about the collagen of sea cucumber except for few reports on *S. japonicus* (Saito, Kunisaki, Urano, & Kimura, 2002) and *Cucumaria frondosa* (Trotter, Lyons-Levy, Thurmond, & Koob, 1995), respectively. However, results of the former finding were not consistent with that of the latter about the molecular composition of the sea cucumber collagen.

In this paper, we tried to describe the characterization of collagen, clarify the molecular composition of that from the sea cucumber *S. japonicus* and identify both similarities and differences between the vertebrate and invertebrate collagen.

2. Materials and methods

2.1. Materials

Six live adult specimens of sea cucumbers *S. japonicus* (average body weight 150 g) were bought from a local

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market in Qingdao during the month of April. All reagents used were of analytical grade.

2.2. Preparation of "crude collagen fibril"

All procedures were performed at 4 °C as previously described (Trotter et al., 1995) with a slight modification. The body wall of *S. japonicus* was dissected free of inner muscular layer with tweezers, cut into small pieces and washed extensively with distilled water. After the samples (100 g wet weight) were stirred in 1 L of distilled water for 30 min, the water was replaced and the extraction in water was repeated once for 1 h. Then the water was replaced with 1 L of 4 mM ethylenediaminetetraacetic acid (EDTA), 0.1 M Tris–HCl, pH 8.0, and stirred overnight. The liquid was decanted and replaced with 1 L of distilled water, in which the samples were stirred slowly for 15 min and the washing steps was repeated twice more. The liquid was then replaced with 500 ml of fresh distilled water and stirred for 2 d. The mixture was centrifuged at 9000*g* for 5 min. The supernatant containing free collagen fibrils was collected and the pellets were stirred with another 500 ml of distilled water after which the steps was repeated. The supernatant was centrifuged at 10,000*g* for 30 min and the precipitate called "crude collagen fibril" was lyophilized using a Christ Freeze Dryer Alpha 1-4 LD (Martin Christ, Osterode am Harz, Germany).

2.3. Isolation of pepsin-solubilized collagen

The following procedures were performed as previously described (Saito et al., 2002) with a slight modification. The "crude collagen fibril" was stirred with 20 volumes (v/w) of 0.1 M NaOH for 3 d in order to remove non-collagenous materials effectively and to exclude the effect of endogenous proteases on collagen (Sato, Yoshinaka, Sato, & Shimizu, 1987). The residue after alkali extraction was thoroughly rinsed with distilled water and then stirred with 10 volumes (v/w) of 0.5 M acetic acid containing porcine pepsin at an enzyme/substrate ratio of 1:100 (w/w). After digestion for 2 d, the suspension was centrifuged at 12,000*g* for 60 min and then the pepsin-solubilized collagen (PSC) in the supernatant was salted out by adding NaCl to a final concentration of 0.8 M. The resultant precipitate collected by low speed centrifugation was dissolved in 0.5 M acetic acid and dialysed against 0.02 mol/l Na_2HPO_4 (pH 8.0) to inactivate pepsin. After several changes of 0.02 mol/l Na_2HPO_4, the precipitate was collected by low speed centrifugation and then dissolved in 0.5 M acetic acid, dialysed against 0.1 M acetic acid for 2 d and lyophilised.

2.4. UV–vis spectra

The UV–vis adsorption spectrum of collagen from *S. japonicus* was recorded using a Shimadzu spectrophotometer UV-2550 (Shimadzu, Tokyo, Japan) from 190 to 400 nm.

2.5. SDS-polyacrylamide gel electrophoresis (SDS-PAGE)

SDS-PAGE was performed as previously described (Laemmli, 1970), using 9% polyacrylamide gels. Collagen samples were dissolved in 0.1 M Na_3PO_4 (pH 7.2) at a concentration of 1 mg/ml. The samples (about 20 μl) were mixed with the sample loading buffer (60 mmol/l Tris–HCl, pH 8.0, containing 25% glycerol, 2% SDS, 0.1% bromophenol blue) at 4:1 (v/v) ratio in the presence or absence of β-ME, then applied to sample wells and electrophoresed, along with type I collagen from calf (Worthington Biochemical Corporation, Lakewood, NJ, USA), low molecular weight markers (Fermentas Life Sciences, Vilnius, Lithuania) and high molecular weight markers (Shanghai Institute of Biochemistry, the Chinese Academy of Sciences, Shanghai, China) in an electrophoresis instrument (Beijing Liuyi Instrument Factory, Beijing, China). The electrophoresis was carried out for about 4 h at a constant voltage of 100 V. After electrophoresis, gel was stained with 0.1% (w/v) Coomassie blue R-250 in 45% (v/v) methanol and 10% (v/v) acetic acid.

2.6. Peptide mapping of PSC

Peptide mapping with endopeptidase Glu-C from *Staphylococcus aureus* strain V-8 (EC.3.4.21.19, Sigma) was performed as previously described (Kittiphattanabawon, Benjakul, Visessanguan, Nagai, & Tanaka, 2005; Mizuta, Miyagi, Nishimiya, & Yoshinaka, 2002; Saito et al., 2002) with a slight modification. The collagen samples from *S. japonicus* (0.4 mg) and calf skin type I collagen (0.4 mg) were dissolved in 0.1 ml of 0.1 M Na_3PO_4 (pH 7.2)containing 0.5% (w/v) SDS. After the addition of 10 μl of the same buffer containing 5 μg of V-8 protease, the reaction mixture was incubated at 37 °C for 25 min, boiled for 3 min, then separated on 9% and 12% polyacrylamide gel in an electrophoresis instrument (Beijing Liuyi Instrument Factory, Beijing, China), and stained as above.

2.7. Amino acid composition

PSC samples were hydrolyzed under reduced pressure using 6 M HCl at 110 °C for 24 h, and the major amino acid composition of hydrolysates was analyzed using a Hitachi amino acid analyzer 835-50 (Hitachi, Tokyo, Japan).

2.8. Differential scanning calorimetry

Differential scanning calorimetry (DSC) was performed on a Netzsch DSC 200 PC (Netzsch, Bavaria, Germany) fitted with a air cooling compressor and a liquid nitrogen cooler at ambient temperature (Schubring, 1997; Schubring, 1999). The instrument was calibrated for temperature and enthalpy using indium, Hg, Bi and Zn as standards. The collagen samples from *S. japonicus* (5 mg) and type I collagen (8 mg) were weighed accurately (0.1 mg) into 30 μl aluminium pans (BO 6.239.2-64.502) and sealed,

respectively. At least triplicate samples were heated from 20 to 70 °C at a scanning rate of 2 K/min, with an empty sealed pan as a reference. The transition temperature (T_{max}) was recorded. The transition enthalpy was determined from the peak area and expressed in J/g of sample.

2.9. Gel filtration chromatography

Gel filtration chromatography was carried out as previously described with a slight modification (Mizuta, Yoshinaka, Sato, Itoh, & Sakaguchi, 1992). The samples (about 6 mg) were stirred in 2 ml of 50 mM Tris–HCl, pH 7.5, containing 2 M urea, 0.1 M NaCl for 2 d at 4 °C and then centrifuged for 30 min at 10,000*g*. The supernatant was denatured at 40 °C for 10 min, then applied to gel filtration column (1.5 × 100 cm) of Sephacryl S-300 HR (Amersham Biosciences, Piscataway, NJ, USA) and eluted at a flow rate of 10 ml/h with 50 mM Tris–HCl, pH 7.5, containing 0.1 M NaCl. Fractions of 2.0 ml were collected and monitored at 220 nm. Appropriate fractions were pooled, dialysed against 0.1 M acetic acid and then lyophilized.

3. Results and discussion

3.1. Preparation and identification of collagen from *S. japonicus*

β-ME was thought to be necessary and classical to isolate collagen fibrils from *Echinodermata* (Matsumura, 1973b; Omura et al., 1996; Robinson, 1997; Saito et al., 2002). Here a different method without β-ME was adopted to isolate collagen fibrils from *S. japonicus* successfully as previously described (Trotter et al., 1995). Incubation of sea cucumber body wall sequentially in water, EDTA, and water as described in Section 2.2 extracted fibrils in large amounts. The method of exposure of sea cucumber body wall to water following chelation of divalent cations suggested the electrostatic interactions to be important in the maintenance of tissue integrity. Contrasting to the vertebrate, collagen fibrils from *S. japonicus* were not nearly solubilized with 0.5 M acetic acid but easily solubilized by limited pepsin proteolysis (Kittiphattanabawon et al., 2005) which was similar to that of other *Echinodermata*.

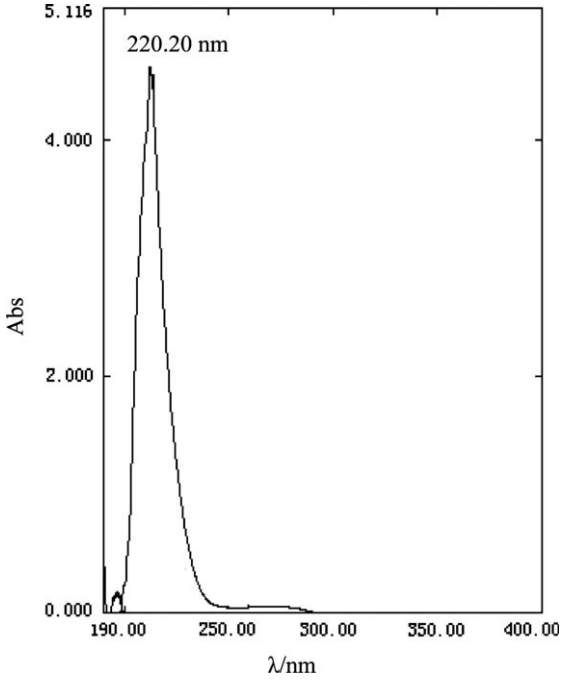

Fig. 1. UV–vis spectrum of PSC (pepsin-solubilized collagen).

The collagen fibrils from *S. japonicus* exhibited a maximum absorbance at 220 nm as shown in Fig. 1, similar to that of type I collagen. This is consistent with the amino acid composition of *S. japonicus* collagen containing few Tyr and Phe with maximum absorbance at 280 nm. The characterization also showed the high purity of PSC.

3.2. The subunit composition of collagen from *S. japonicus*

Fig. 2(a) shows SDS-PAGE patterns of PSC with a 1α trimer (about 135 kDa) while 1α chain resembled α_1 chain of type I collagen of vertebrate. The addition of β-ME did not affect the SDS-PAGE patterns, suggesting the

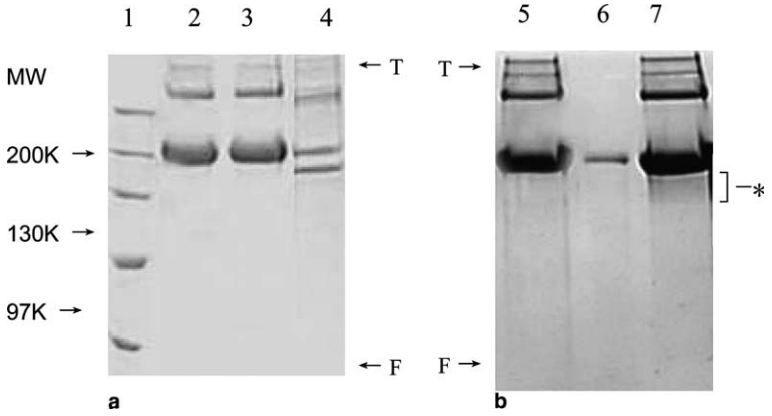

Fig. 2. SDS-PAGE patterns of PSC (pepsin-solubilized collagen), high molecular weight markers and type I collagen. 1, 2, 3, 4, 5, 6, 7 denoted high molecular markers, PSC (the sample solution without mercaptoethanol), PSC (the sample solution with mercaptoethanol), type I collagen, PSC, 1α chain of PSC excised from (a) and PSC after DSC analysis, respectively. T: gel top and F: buffer front.

absence of molecular species with disulphide bonds (as shown in band 3 of Fig. 2(a)). Together with the results of precipitation properties by NaCl under acidic pH and SDS-PAGE, collagen of *S. japonicus* might be classified as type I collagen. As for the subunit composition, Saito et al. (2002) reported that collagen molecules from *S. japonicus* had the subunit composition of $(\alpha_1)_2\alpha_2$.

To elucidate the subunit composition of collagen molecules of *S. japonicus*, we performed two-dimensional gel electrophoresis. After the first dimensional gel electrophoresis, the gels with α_1 chains which had not been stained were excised, cut into pieces and soaked in 0.1 M Na_3PO_4 (pH 7.2) for 2 days at 4 °C. After centrifugation, the supernatant with α_1 chains were applied to the sample wells and performed the second dimensional gel electrophoresis (Fig. 2(b)) which was similar to the first dimensional gel electrophoresis. As shown in Fig. 2(b), band 6 showed the same electrophoresis pattern as the 1α chain of band 5. Thus, the subunit structure of $(\alpha_1)_3$ appeared to be evident. The results were consistent with the reports of Trotter et al. (1995) about the subunit composition of collagen molecules of sea cucumber *C. frondosa*. To further identify the subunit composition of collagen, isolation of α chains was achieved by gel filtration on Sephacryl S-300 HR. A single peak with shoulder was observed in Fig. 3. Then the different fractions of the peak were collected for SDS-PAGE (the inset of Fig. 3). As shown in the inset, the fraction d contained only a single α chain that shared the same electrophoresis pattern as α chain of PSC. Together with the results of SDS-PAGE and gel filtration, however, the presence of $(\alpha_1)_3$ trimers was evident.

The subunit structure of $(\alpha_1)_3$ exists in the invertebrate extensively (Kimura & Tanaka, 1983; Mizuta et al., 1992). The sea urchin collagen molecules were thought to be composed of two α_1 and one α_2 chains (Omura et al., 1996; Robinson, 1997), however, Cluzel et al. (2000) found that the structure to be $(\alpha_1)_3$ in sea urchin. Here, we could define that *S. japonicus* collagen molecules are composed of three α_1 chains. When compared to our present results, it seems that α_2 chains of the results of Saito et al. (2002) were derived from the degradation products of α_1 due to the treatment with pepsin.

3.3. Peptides of collagen from S. japonicus and type I collagen

The peptide maps of PSC digested by V8 protease, in comparison with calf skin type I collagen, are shown in Fig. 4. PSC digested by V8 protease showed better electrophoresis patterns on 12% gel than that on 9% gel (data not shown). Judging from the peptide maps of V8 digests, γ- and β-compositions of type I collagen slightly degraded, however, α- and molecular cross-linked components of PSC were markedly degraded after digestion with V8 protease. These results suggested that calf skin type I collagen was more tolerant to digestion by V8 protease than PSC. V8 protease exhibited a high degree of specificity for glutamic acid and aspartic acid residues and proteins (Kittiphattanabawon et al., 2005). Thus, glutamic acid and aspartic acid residues of skin collagen of calf might be lower than that of PSC, which is consistent with the results of amino acid analysis listed in Table 1. After digestion, PSC was degraded into three major peptide fragments with molecular weights of 50, 30 and 20 kDa.

3.4. Correlation between amino acid compositions and thermal stability

The amino acid compositions of body wall protein of *S. japonicus* and PSC are shown in Table 1. The compositional

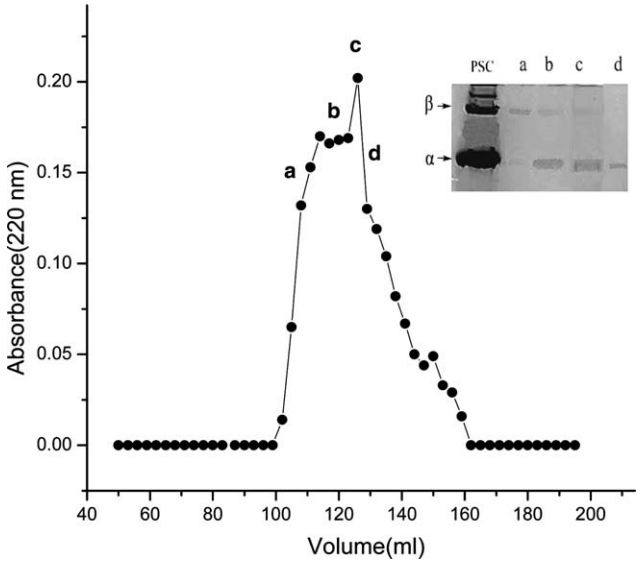

Fig. 3. Gel filtration chromatography of PSC (pepsin-solubilized collagen) on a column (1.5 × 100 cm) of Sephacryl S-300 HR. The inset shows SDS-PAGE, Coomassie blue-stained, of PSC, a–d of the peak.

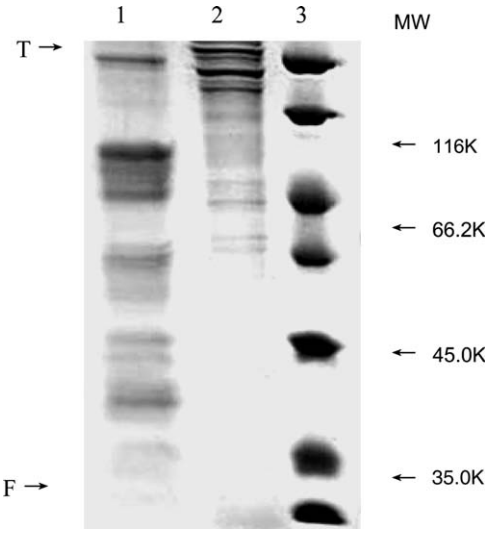

Fig. 4. Peptide maps of V8 digests of PSC (pepsin-solubilized collagen) and type I collagen on 12% gel. 1, 2, 3 denoted peptide map of PSC, type I collagen and low molecular markers. T: gel top and F: buffer front.

Table 1
Amino acid compositions of sea cucumber body wall and PSC (pepsin-solubilized collagen) compared with calf skin collagen (residues per 1000 residues)

Amino acid	*Stichopus japonicus*		Calf skin collagen[a]
	Body wall	PSC (pepsin-solubilized collagen)	
Asp	75.8 ± 2.0	59.5 ± 1.5	45
Thr	43.7 ± 0.8	34.3 ± 1.0	18
Ser	49.4 ± 1.2	44.6 ± 1.2	39
Glu	105.6 ± 3.0	103.9 ± 2.5	75
Gly	265.4 ± 10.0	328.7 ± 5.0	330
Ala	96.5 ± 6.0	110.6 ± 4.5	119
Val	34.4 ± 0.9	24.3 ± 0.5	21
Met	15.4 ± 1.0	9.0 ± 0.5	6
Ile	24.6 ± 3.0	18.4 ± 1.0	11
Leu	32.0 ± 4.0	18.6 ± 0.5	23
Tyr	21.2 ± 3.0	8.2 ± 0.2	3
Phe	21.2 ± 3.0	7.4 ± 0.2	3
Lys	23.0 ± 2.3	5.0 ± 0.2	26
His	7.1 ± 0.5	3.0 ± 0.1	5
Arg	47.8 ± 5.0	53.2 ± 3.2	50
Pro	75.7 ± 6.5	94.7 ± 4.5	121
Hyp	52.2 ± 5.0	66.3 ± 4.2	94
Hyl	9.0 ± 0.5	10.3 ± 0.1	7

The mean ± standard deviation of three determinations for the same sample preparations.
[a] Li et al. (2004).

features of PSC indicates high content of glycine (328.7 residues/1000) and hydroxyproline (66.3 residues/1000), but low contents of tyrosine, phenylalanine and histidine. No cysteine was detected. The relatively high contents of characteristic amino acids, glycine, hydroxyproline and hydroxylysine of the body wall protein suggested collagen as the major protein component. The ratio of hydroxyproline to proline was 0.70. The distribution patterns of amino acid composition were closer to that of skin collagen of calf which indicates that PSC might be classified as type I collagen.

Fig. 5 shows the differential scanning calorimetric thermograms of type I collagen (1) and PSC (2). The thermal stability (T_s) temperature of PSC was 57.0 °C, about 5.0 °C lower than that of type I collagen of calf. Ts depends on the degree of hydratation and hydroxyproline content

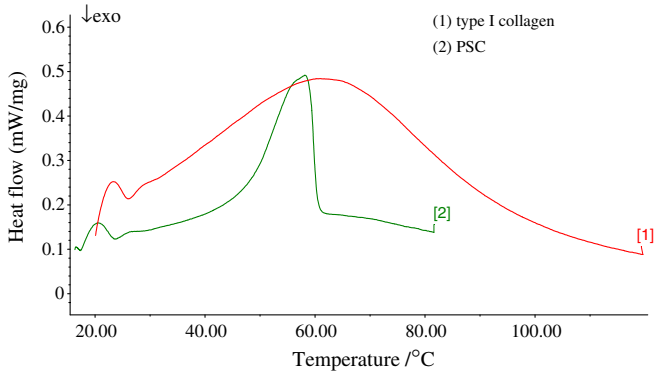

Fig. 5. DSC 200 PC measured curve of type I collagen (1) and PSC (pepsin-solubilized collagen, 2).

(Sionkowska & Kaminska, 1999). Our results about the correlation between amino acid compositions and T_s also demonstrated the one conclusion. As shown in Table 1, proline and hydroxyproline contents of PSC were about 78.3% and 70.5% of that of type I collagen, respectively, which is consistent with the results of T_s. To probe into the structural change of protein after denaturation, PSC after DSC analysis was performed on SDS-PAGE as shown in band 7 of Fig. 2(b). Contrasting to the native PSC (band 5), the denatured PSC (band 7) showed slight degradation products from α_1 chains (as denoted by the signal *). This phenomenon suggests that the peptide bonds could be ruptured at temperatures higher than T_s. Thus, one needs a low temperature when preparing collagen.

4. Conclusions

Collagens prepared from sea cucumber *S. japonicus* were classified as type I collagen with the molecular composition of $(\alpha_1)_3$. T_s and peptide maps of collagen from *S. japonicus* digested by V8 protease were different from that of type I collagen due to some differences in amino acid contents and sequence.

Acknowledgments

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