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ARTICLE

# Purification and Characterization of Pepsin-Solubilized Collagen from Skin and Connective Tissue of Giant Red Sea Cucumber (*Parastichopus californicus*)

Zunying Liu,[†] Alexandra C. M. Oliveira,[‡] and Yi-Cheng Su*[,§]

[†]College of Food Science and Engineering, Ocean University of China, 5 Yushan Road, Qingdao, Shandong Province 266003, People's Republic of China, [‡]Fishery Industrial Technology Center, University of Alaska Fairbanks, 118 Trident Way, Kodiak, Alaska 99615, and [§]Seafood Research and Education Center, Oregon State University, Room 253, 2001 Marine Drive, Astoria, Oregon 97103

Pepsin-solubilized collagen (PSC) was extracted from giant red sea cucumbers (*Parastichopus californicus*) and characterized for denaturation temperature ($T_d$), maximum transition temperature ($T_m$), enzyme-digested peptide maps, and gel-forming capability. SDS-PAGE showed that PSCs from giant red sea cucumber skin and connective tissue were both type I collagens, consisting of three $\alpha_1$ chains of approximately 138 kDa each. The amino acid composition and peptide maps of PSCs digested by V8 protease were different from those of calf skin type I collagen. The $T_d$ and $T_m$ are 18.5 and 33.2 °C, respectively, for skin PSC and are 17.9 and 32.7 °C, respectively, for connective tissue PSC. Both skin and connective tissue PSCs exhibited good gel-forming capability at pH 6.5 and at an ionic strength of 300 mM salt (NaCl). Collagen isolated from giant red sea cucumbers might be used as an alternative to mammalian collagen in the food and pharmaceutical industries.

**KEYWORDS: Collagen; giant red sea cucumber; *Parastichopus californicus*; characterization; thermal stability**

## INTRODUCTION

Collagen is an abundant protein in animal tissues and has a wide range of applications in the biomedical, pharmaceutical, cosmetic, and food industries (*1*). The global demand for collagen has been increasing over the years. However, the industrial use of collagen has been limited to vertebrate collagens, most of which were produced from pig skin (80%), followed by cattle split (15%) and other sources such as pig and cattle bones, poultry, and fish (5%) (*2*). More than 27 types of collagen have been identified from various animal and human tissues, with each type having a distinctive amino acid sequence and molecular structure to play a unique role in the tissue (*3*).

Despite its wide range of applications, the use of collagen in food products may be a concern among consumers because of dietary restriction and concerns over bovine spongiform encephalopathy (BSE) and transmissible spongiform encephalopathy (TSE). Therefore, new sources for collagen are being explored, and marine organisms have been recognized as potential resources due to their availability, the lack of dietary restriction or risk of disease transmission, and the possibility of high collagen yields (*4*). Collagens with different properties have been isolated from several marine animals, such as squid (*Illex argentinus*), Japanese sea-bass (*Lateolabrax japonicus*), oyster (*Pinctada fucata*), and cuttlefish (*Sepia lycidas*) (*5*−*8*). However, the isolation of collagen from a commercially important giant red sea cucumber (*Parastichopus californicus*) has not been explored.

*Corresponding author [telephone (503) 325-4531; fax (503) 325-2753; e-mail yi-cheng.su@oregonstate.edu].

The giant red sea cucumber is the largest member (more than 12 in. in length) of the family Holothuroidea and is the largest sea cucumber species along the Pacific Northwest coast. Sea cucumbers are commercially harvested by divers in Alaska (300,000 to 400,000 kg/year) and sold to Asian markets as trepang (brined and dehydrated skin) and frozen meat (*9*). Although the skin of the giant red sea cucumber can be marketed as trepang, it contains a high content of collagen and may be better utilized as a resource of collagen for nutraceutical and pharmaceutical applications. Therefore, the objective of this study was to isolate and partially characterize pepsin-solubilized collagens (PSCs) from skin and connective tissue of giant red sea cucumber (*P. californicus*).

## MATERIALS AND METHODS

**Materials.** Giant red sea cucumbers (*P. californicus*) were hand-picked by divers off Kodiak Island (Alaska) during the commercial fishing season from October 2008 to February 2009. The skin and connective tissue (tissue between skin and longitudinal muscle strips) were removed from the sea cucumber immediately upon delivery of the live animals to the pilot plant of the Fishery Industrial Technology Center of University of Alaska Fairbanks. The skin and connective tissue samples were then vacuum-packed and shipped overnight to the Seafood Research and Education Center of Oregon State University. The samples were kept frozen at −80 °C until analysis.

**Proximate Analysis.** The moisture, lipid, ash, and protein contents of skin and connective tissue from giant red sea cucumbers were determined according to AOAC methods (*10*). A conversion factor of 6.25 was used to calculate the protein contents in samples from the Kjeldahl nitrogen contents. All analyses were conducted in triplicates.

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**Isolation and Purification of Pepsin-Solubilized Collagen.** The collagen in sea cucumber skin and connective tissue was extracted as previously described (*11*, *12*) with some modifications. All steps were performed at 4 °C with continuous stirring. Skin was cut into small pieces and washed in distilled water with continuous stirring for 30 min. The washing process was repeated twice. Washed skin (100 g wet weight) was stirred in 1 L of 4 mM ethylenediaminetetraacetic acid (EDTA) solution containing 0.1 M Tris-HCl (pH 8.0) for 24 h. The samples were transferred to 1 L of distilled water and stirred for 24 h. The mixture was poured through a cheesecloth to remove water-insoluble components. Free collagen fibrils in the filtrate were centrifuged at 9000*g* at 4 °C for 30 min. The precipitate (50−70 mL) was mixed with 20 volumes of 0.1 M NaOH and stirred for 72 h to dissolve noncollagen materials. The collagen was recovered by centrifugation at 9000*g* at 4 °C for 30 min. The insoluble component was washed with distilled water several times until the pH reached about 7.0, which was followed by solubilization in 20 volumes of 0.5 M acetic acid containing 0.5% (w/w) pepsin (EC 3.4.23.1, Sigma-Aldrich Inc., St. Louis, MO) with continuous stirring for 48 h. The solution was centrifuged at 12000*g* at 4 °C for 60 min, and supernatant was collected. The precipitate was dispensed into 20 volumes of 0.5 M acetic acid containing 0.5% (w/w) pepsin to extract the remaining collagen as described above. Collagen in the supernatant was salted out by adding 0.8 M NaCl and stirring for 24 h. The crude collagen was collected by centrifuging at 9000*g* for 30 min, dissolved in 0.5 M acetic acid, and dialyzed against 0.02 M $Na_2HPO_4$ and 0.1 M acetic acid. The purified PSC was lyophilized and stored in a refrigerator for 1−3 weeks until analysis.

**SDS-PAGE.** SDS-PAGE was performed according to the method of Laemmli (*13*) using a discontinuous Tris-HCl/glycine buffer system with 7.5% resolving gel and 4% stacking gel. The collagen samples were dissolved in a sample buffer (0.06 M Tris-HCl, pH 6.8, containing 2% SDS, 25% glycerol, 0.1% bromophenol blue) and then boiled for 3 min. Electrophoresis was conducted using the Mini PROTEAN 3 Cell (Bio-Rad Laboratories Inc., Richmond, CA) at 120 V. After electrophoresis, gels were stained for 30 min with 0.1% Coomassie brilliant blue R-250 solution followed by destaining in a solution containing distilled water, methanol, and acetic acid at a ratio of 8:1:1 (v/v/v). SDS, glycerol, bromophenol blue, Coomassie brilliant blue R-250, and SDS-PAGE standards were purchased from Bio-Rad Laboratories.

**Peptide Mapping of PSC.** Peptide mapping of PSC from the giant red sea cucumber was conducted with endopeptidase Glu-C from the *Staphylococcus aureus* strain V-8 (EC 3.4.21.19, Sigma-Aldrich), as described by Cui and others (*14*), with a slight modification. The PSC samples and calf skin type I collagen (Sigma-Aldrich) were dissolved in 0.1 mL of 0.1 M sodium phosphate buffer (pH 7.8) containing 0.5% (w/v) SDS instead of 0.1 M $Na_3PO_4$ solution (pH 7.2). An aliquot (10 $\mu$L) of the sodium phosphate buffer (pH 7.8) containing 5 $\mu$g of V-8 protease (250−500 units/mg) was then added to the sample mixture and incubated at 37 °C for 25 min. The digestion reaction was terminated by heating the reaction mixture in boiling water for 3 min. Peptides generated from the protease digestion were analyzed by SDS-PAGE with 15 and 16.5% gels, as described earlier.

**Amino Acid Composition of PSC.** The amino acid composition of PSC was determined as previously described (*14*). PSC samples were hydrolyzed under vacuum with 6 M HCl at 110 °C for 24 h, and the major amino acid composition of hydrolysates was analyzed by a commercial laboratory using a Hitachi amino acid analyzer L-8900 (Hitachi, Tokyo, Japan).

**Determination of Denaturation Temperature.** The denaturation temperature ($T_d$) of PSC from the giant red sea cucumber was measured from viscosity changes using an Ubbelohde viscosimeter (Ubb-2C, Technical Glass Products, Inc., Painesville, OH). The freeze-dried collagen samples were dissolved in 0.1 M acetic acid to a protein concentration of 0.3 mg/mL. The viscosity of the collagen solution (containing protein at 0.3 mg/mL) was determined by measuring the efflux time ($t$) of the solution after incubation of the collagen solution (10 mL) at given temperatures between 10 and 45 °C for 30 min. The efflux time of 0.1 M acetic acid ($t_0$) was determined at the same temperature as a control. The fractional viscosity at a given temperature [$F(T)$] was calculated according to the equation $F(T) = (\eta_{sp(T)} - \eta_{sp(45°C)})/(\eta_{sp(10°C)} - \eta_{sp(45°C)})$, where $\eta_{sp}$ is the specific viscosity at $T$ temperature and is calculated as $(t - t_0)/t_0$. Calculated fractional viscosities were plotted against temperatures, and the denaturation temperature was determined at the level where the fractional viscosity equaled 0.5.

**Table 1.** Proximate Composition of Giant Red Sea Cucumber Skin and Connective Tissue (on Wet Weight Basis)[a]

| | moisture (%) | lipid (%) | ash (%) | protein (%) |
|---|---|---|---|---|
| skin | 90.14 ± 0.14a | 0.57 ± 0.04a | 2.83 ± 0.05a | 4.94 ± 0.47b |
| connective tissue | 85.66 ± 0.29b | 0.44 ± 0.02a | 1.72 ± 0.04b | 9.46 ± 0.08a |

[a] Values are means ± standard deviation ($n$ = 3). Means with different letters in the same column are significantly different ($P$ < 0.05).

**Thermal Transition of PSC.** The maximum transition temperature ($T_m$) was determined using a micro-DSC III calorimeter (SETARAM, Caluire, France). The freeze-dried collagen powder was dissolved in deionized water to prepare a collagen solution containing protein of 40 mg/mL. The collagen solution was held at 4 °C for 24 h, and 500 mg of the solution was weighed accurately and sealed into vessels. The enthalpy of a sample was recorded from 20 to 80 °C at a heating rate of 1 °C/min under a nitrogen atmosphere using deionized water as a reference. $T_m$ was estimated from the differential scanning calorimetric (DSC) thermogram.

**Gel-Forming Capacity of PSC.** The gel-forming capacity of PSC of giant red sea cucumber was performed according to procedures from Shen and others (*15*) with a slight modification. The freeze-dried PSC was dissolved in 0.1 M acetic acid instead of pH 3.0 HCl solution to obtain a concentration of 0.5% (w/v). The effects of pH values on gel formation from the PSC were determined by mixing 2 mL of PSC solution with 2 mL of 0.01 M sodium phosphate buffer containing 100 mM NaCl at various pH values (5.5, 6.0, 6.5, 7.0, 7.5, and 8.0). The effects of salt (NaCl) concentrations on gel formation from the PSC were observed by mixing 2 mL of PSC solution with 2 mL of 0.01 M sodium phosphate buffer (pH 7.0) containing various concentrations of NaCl (0, 50, 100, 150, 200, 300, and 500 mM). The mixtures were kept at 4 °C for 30 min. Gel formation was monitored by measuring the absorbance value at 310 nm for the mixture based on turbidity change.

**Statistical Analysis.** All experiments were replicated three times, and each experiment was conducted with three determinations. Data were analyzed by one-way analysis of variance (ANOVA). Mean separations were performed by Duncan's new multiple-range test (Excel, Microsoft). Significant differences between means were established at a level of $P$ = 0.05. Samples in the first replicate were used for analysis of peptide mapping and amino acid composition of PSC.

## RESULTS AND DISCUSSION

**Proximate Analysis.** The proximate compositions (on wet weight basis) for skin and connective tissue of giant red sea cucumber are shown in **Table 1**. Both skin and connective tissue contained very low amounts of lipid (0.44−0.57%). The skin contained higher ($P$ < 0.05) moisture (90.1%) and ash (2.83%) than did connective tissue (85.7% moisture and 1.72% ash). However, protein contents were considerably lower ($P$ < 0.05) in skin (4.94%) than in connective tissue (9.46%) because of the higher moisture content ($P$ < 0.05) of skin. Muyonga and others (*16*) reported that the ash content was higher in perch fish skin because of increased mineralization with age. It is possible that the giant red sea cucumber also accumulates minerals in its skin similarly to perch fish. However, such a speculation remains to be investigated.

As a whole, results of this study showed that the proximate composition of giant red sea cucumber tissues are similar to those reported for the California sea cucumber (*P. californicus*) (*17*) and the sea cucumber *Holothuria tubulosa* from the Turkish Sea (*18*). Additionally, in previous studies the protein content of freeze-dried giant red sea cucumber muscle strips was higher (66%) than in their freeze-dried skin counterparts (47%) (*19*). Therefore, the proximate composition of the connective tissue is similar to the composition of the muscle strips rather than the skin.

**Isolation and Purification of PSC.** The giant red sea cucumber contains little acid-soluble collagen (ASC). The yield of acid-soluble collagen (ASC) from skin was only 3.4% on a dry weight

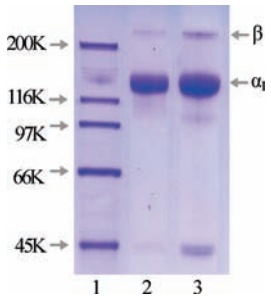


**Figure 1.** SDS-PAGE pattern of pepsin-solubilized collagen (PSC) from giant red sea cucumber skin and connective tissue on 7.5% gel. Lanes: 1, protein markers; 2, skin PSC; 3, connective tissue PSC.

basis. However, collagen in the skin and connective tissue of giant red sea cucumbers could be isolated by pepsin digestion procedures with yields of 20.8% from skin and 24.3% from connective tissue on a dry weight basis. The yields of collagen from giant red sea cucumbers were lower than those from channel catfish (*Ictalurus punctatus*) skin (38.4%) (*4*) and grass carp (*Ctenopharyngodon idella*) skin (46.6%) (*20*) but were similar to that from largefin longbarbel catfish (*Mystus macropterus*) skin (28.0%) (*21*). The difference in yields (on a dry weight basis) of ASC (3.4%) and PSC (20.8%) from the skin of giant red sea cucumber suggests that interchain cross-linkages exist in the telopeptide region of the collagen, which makes the collagen less soluble under an acidic condition. Such a cross-linkage could be removed by pepsin digestion without damaging the integrity of the triple helix (*20*). Therefore, increased yields of collagen from skin of giant red sea cucumber were observed using pepsin digestion procedures.

**SDS-PAGE and Peptide Mapping of PSC.** SDS-PAGE analysis of PSC from the skin and connective tissue of giant red sea cucumbers revealed that both consisted of a major component ($\alpha_1$) of approximately 138 kDa and a small amount of dimers ($\beta$ chains) (**Figure 1**). No apparent difference was noted in the SDS-PAGE between the PSC from skin and connective tissue. The SDS-PAGE patterns ($\alpha_1$ and $\beta$ dimer) of the PSC from giant red sea cucumber are similar to those reported for collagens from other sea cucumber species (*11*, *14*). This observation indicates that collagens from giant red sea cucumber appear to be type I collagen. Kimura and Ohno (*22*) reported a unique $\alpha_1\alpha_2\alpha_3$ heterotrimer consisting of three nonidentical chains in Alaska pollack skin collagen, which indicated the existence of $\alpha_3$ chain in Alaska pollack skin collagen. However, it is not clear if the $\alpha_3$ chain exists in the collagens of giant red sea cucumber. The $\alpha_3$ chain is known to have a chemical nature similar to that of the $\alpha_1$ chain and will not be separated by SDS-PAGE because both $\alpha_1$ and $\alpha_3$ chains migrate electrophoretically on the gel to similar positions (*23*).

The peptide maps of PSC from skin and connective tissue of giant red sea cucumber digested by V8 protease were compared with that of calf skin type I collagen by SDS-PAGE using 16.5 and 15% gels (**Figure 2**). Enzyme-digested PSC from giant red sea cucumber skin and connective tissue contained only one major peptide fragment with a molecular mass of 40 kDa when analyzed on a 15% gel. However, several peptide fragments with molecular masses of about 200 kDa were obtained after the enzyme digestion of calf skin type I collagen. These results suggested that calf skin type I collagen was more resistant than PSC from giant red sea cucumbers to V8 protease digestion and that the primary structures of collagen from giant red sea cucumbers may be different from that of calf skin collagen.

Analysis of peptide maps of PSC from giant red sea cucumbers digested by V8 protease indicated that the PSC from the connective tissue appeared to be more resistant to the enzyme digestion than was the PSC from skin (**Figure 2**). Nearly all of the PSC from skin was degraded by the enzyme to small peptide fragments with molecular masses of lower than 45 kDa. However, peptide fragments of molecular masses between 45 and 60 kDa were found in the PSC from connective tissue after enzyme digestion. The difference in the peptide maps of PSC between skin and connective tissue of the giant red sea cucumber digested by V8 protease is probably due to the difference in accessibilities of susceptible bonds to the protease, which leads to diverse patterns of hydrolysis between the PSC from skin and connective tissue.

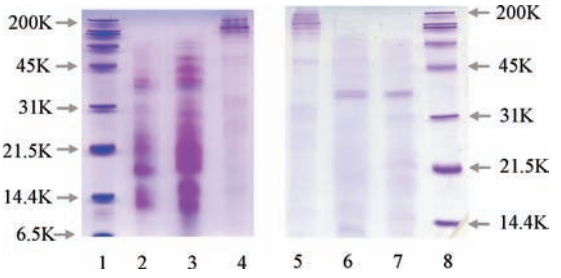


**Figure 2.** SDS-PAGE analysis of peptide maps of pepsin-solubilized collagen from giant red sea cucumber skin and connective tissue digested by V8 protease on 16.5% (lanes 1−4) and 15% (lanes 5−8) gels. Lanes: 1 and 8, protein markers; 2−4, skin PSC, connective tissue PSC, and calf skin collagen, respectively; 5−7, calf skin collagen, connective tissue PSC, and skin PSC, respectively.

**Table 2.** Comparison of Amino Acid Composition of Pepsin-Solubilized Collagen (PSC) from Giant Red Sea Cucumber Skin and Connective Tissue with Amino Acid Composition of PSC from Body Wall of Sea Cucumber (*Stichopus japonicus*) (Residues/1000 Residues)

| | giant red sea cucumber PSC | | body wall PSC (*S. japonicus*) | |
|---|---|---|---|---|
| amino acid[a] | skin | connective tissue | Saito and others (*12*) | Cui and others (*14*) |
| hydroxyproline (Hyp) | 58 | 52 | 68 | 66 |
| hydroxylysine (Hyl) | nd[b] | nd | 10 | 10 |
| aspartic acid (Asp) | 77 | 76 | 59 | 60 |
| threonine (Thr) | 45 | 48 | 33 | 34 |
| serine (Ser) | 65 | 65 | 44 | 45 |
| glutamic acid (Glu) | 119 | 111 | 109 | 104 |
| glycine (Gly) | 332 | 303 | 325 | 329 |
| alanine (Ala) | 99 | 92 | 112 | 111 |
| valine (Val) | 30 | 31 | 23 | 24 |
| methionine (Met) | nd | nd | 9 | 9 |
| isoleucine (Ile) | 23 | 25 | 21 | 18 |
| leucine (Leu) | 25 | 28 | 18 | 19 |
| tyrosine (Tyr) | 10 | 12 | 5 | 8 |
| phenylalanine (Phe) | 10 | 14 | 3 | 7 |
| lysine (Lys) | 7 | 6 | 7 | 5 |
| histidine (His) | 4 | 3 | 4 | 3 |
| arginine (Arg) | 50 | 43 | 55 | 53 |
| proline (Pro) | 95 | 90 | 95 | 95 |
| total | 1000 | 1000 | 1000 | 1000 |
| imino acid | 153 | 142 | 163 | 161 |

[a] Methionine and cysteine were destroyed in acid hydrolysis and were not determined separately. [b] Not determined.

**Amino Acid Composition.** Amino acid analysis of PSC from the skin and connective tissue of giant red sea cucumbers revealed that both PSCs were rich in glycine, alanine, proline, and glutamic acid (**Table 2**). These results are similar to the amino acid composition reported for collagen from a commercially important sea cucumber species (*Stichopus japonicus*) in Japan (*12*). Compared with PSC from connective tissue, PSC from skin

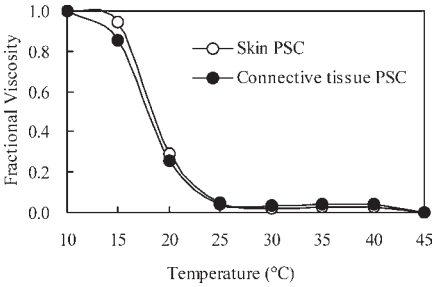


**Figure 3.** Thermal denaturation curve of pepsin-solubilized collagen (PSC) from giant red sea cucumber skin and connective tissue.

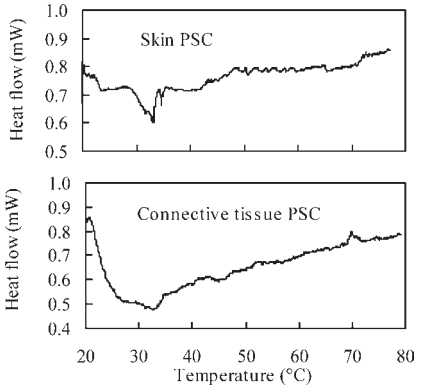


**Figure 4.** Thermal transition curve of pepsin-solubilized collagen (PSC) from giant red sea cucumber skin and connective tissue.

contained higher amounts of glycine, hydroxyproline, proline, and arginine but lower amounts of leucine and phenylalanine. The imino acid (proline + hydroxyproline) contents of PSC from skin and connective tissue were 153 and 142 residues/1000 total amino acid residues, respectively, which were lower than those reported for walleye pollack (184 residues/1000 residues) (*24*) and cod (175.6 residues/1000 residues) (*25*), but were similar to those reported for sea cucumber's (*S. japonicus*) body wall collagen (161 residues/1000 residues) (*14*). The proline and hydroxyproline content in the collagen of a species may vary depending on its living habitat (*26*). The pyrolidine rings of proline and hydroxyproline impose restrictions on the conformation of the polypeptide chain and help strengthen the triple-helix structure (*20*). Thus, collagen from giant red sea cucumbers might have a molecular conformation different from those of walleye pollack and cod collagens owing to the low content of imino acids.

**Thermal Stability.** The thermal stability of collagen is commonly described by its denaturation temperature ($T_d$) and the maximum transition temperature ($T_m$). **Figure 3** shows that the fractional viscosity of PSC from giant red sea cucumber skin and connective tissue in both cases decreased as temperature increased due to protein denaturation. PSC from both sources exhibited a rapid loss of viscosity when heated from 10 to 25 °C. The $T_d$ values of PSC from skin and connective tissue were calculated to be 18.5 and 17.9 °C, respectively; these figures were lower by about 18.5 and 19.1 °C than that of porcine skin collagen ($T_d$ = 37.0 °C) (*27*). The thermal transition curve of PSC from skin and connective tissue in deionized water is shown in **Figure 4**. The $T_m$ values of PSC from skin and connective tissue (33.2 and 32.7 °C, respectively) are lower by about 7.6 and 8.1 °C than that of calf skin collagen ($T_m$ = 40.8 °C) (*28*) but similar to that of collagen from the skin of silver carp (*Hypophthalmichthys molitrix*) (*29*). These results demonstrate that the helices of collagen from giant red sea cucumber were less stable than those of mammalian collagens.

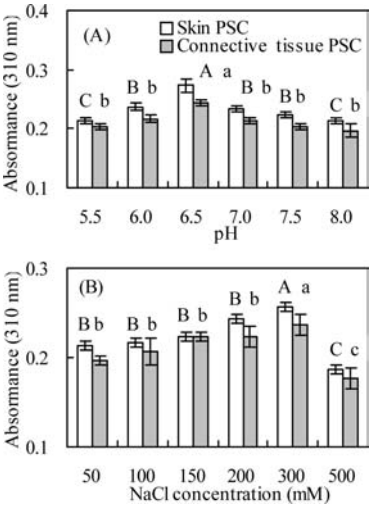


**Figure 5.** Effect of pH (**A**) and NaCl concentration (**B**) on giant red sea cucumber collagen gel formation at 4 °C. Gel formation was evaluated by measuring absorbance at 310 nm based on turbidity change. Values are means ± standard deviation ($n$ = 3). Means with a different letters are significantly different ($P < 0.05$).

The thermal stability of collagen is influenced by imino acid content. Hydroxyproline plays an important role in the stabilization of the collagen triple-helix structure due to its hydrogen-bonding ability through its −OH group (*20*). Therefore, a collagen with high imino acid content typically exhibits a higher denaturation temperature. The imino acid content of calf skin collagen has been reported to be 215 residues/1000 residues (*14*). Compared with that, the imino acid contents of collagens from giant red sea cucumber skin (153 residues/1000 residues) and connective tissue (142 residues/1000 residues) are considered to be low. This is why the $T_d$ of collagens from giant red sea cucumber is lower than that of porcine skin collagen. However, this result was in agreement with a previous report that the thermal stability of collagen of an organism was correlated with environmental and body temperature (*26*). The $T_d$ of collagen from deep-sea giant red sea cucumber, which perches at ocean temperatures of 3−8 °C, was similar to that of coldwater fish such as Argentine hake (*Merluccius hubbsi*) (10 °C) (*30*) and Alaska pollock (*Theragra chalcogramma*) (16.8 °C) (*22*).

**Gel-Forming Ability.** The ability of PSC from giant red sea cucumber to form gel at 4 °C was greatly influenced by ionic strength and pH (**Figure 5**). The optimal condition for gel formation was observed at an ionic strength of 300 mM NaCl or at pH 6.5. However, the calf skin collagen was found to have a greater ability than giant red sea cucumber collagen to form gel under the same condition (data not shown). The lower gel-forming ability of collagen from giant red sea cucumber might be due to the low content of hydroxyproline, which is considered to be one of the main factors affecting the formation of the three-dimensional branched network (*31*). These results also imply that the habitat and tissue structure can affect the gel-forming capability of collagens from different species.

This study demonstrated that collagens from giant red sea cucumber skin and connective tissue were type I collagens with slightly different amino acid compositions and thermal stabilities. The peptide maps of collagen from giant red sea cucumber skin and connective tissue digested by V8 protease were different from those of calf skin type I collagen. These collagens showed relative thermal stability with good gel-forming ability at pH 6.5. These results suggest that giant red sea cucumber collagen holds promising potential as a candidate alternative to mammalian collagen for application in future industries.

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