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Article

Biochemical Characterization of Collagen from the Starfish *Asterias amurensis*

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The industrial use of bovine and porcine collagen has been expanded to include foods, cosmetics, and biomaterials. However, due to outbreaks of bovine spongiform encephalopathy and foot and mouth disease, new, alternative, safe sources of collagen are being sought. In this study, pepsin-solubilized collagen (PSC) was isolated from the tissue of a starfish (*Asterias amurensis*) and shown to consist of $(\alpha 1)_2\alpha 2$ heterotrimer, which is similar to calf skin type I collagen. However, the PSC was denaturated at 24.7°C, which is about 12°C lower than mammalian collagen. Immunoblotting assay using polyclonal anti-type I collagen antibody revealed that the starfish collagen contained similar affinity motifs. In XTT assay, PSC suspension had cell growth activity and no showed no cytotoxicity.

Key words: *characterization, collagen, immunoblotting assay, starfish, XTT assay*

Collagen is a common protein in the living body and a major constituent of connective tissue. It has potential use in a wide range of applications in the pharmaceutical, cosmetic, biomedical, and food industries. Collagen is considered to be one of the most useful biomaterials. Its excellent biocompatibility and high degree of safety are due to its biodegradability and weak antigenicity [Maeda *et al*., 1999]. Examples of collagen application in medicine include its use as a collagen shield in ophthalmology [Kaufman *et al*., 1994], sponge for burns/wounds [Rao, 1995], basic matrix for cell culture systems in tissue engineering [Kemp, 2000], and surgical suture [Miller *et al*., 1964]. Generally, the skin and bones of pigs and cows are the main sources of collagen for the preparation of gelatin. However, outbreaks of bovine spongiform encephalopathy and foot-and-mouth disease have caused anxiety among users of cattle gelatin. Additionally, collagen obtained from pig bones cannot be used in some areas because of religious reasons. Accordingly, there is an increasing need for alternative, safe sources of collagen [Sadowska *et al*., 2003].

In the literature, there have been many studies on the practical utilization of the connective tissue and skin of marine vertebrates and invertebrates such as starfish, squid, jellyfish, sea urchin, bigeye snapper, and skate [Kimura *et al*., 1993; Mizuta *et al*., 1994; Nagai and Suzuki, 2000; Nagai *et al*., 2000; Mizuta *et al*., 2002; Kittiphattanabawon *et al*., 2005]. The starfish *Asterias amurensis* has been considered for aquaculture of shellfish. The body wall of *A. amurensis* is known to be rich in collagen fibers, which can be dissolved into fibrous components [Matsumura, 1973]. Despite the recognized importance of collagen, only a few studies have been undertaken on the molecular characterization of collagen derived from the starfish *A. amurensis*.

The objective of this research, then, is to investigate the preparation, biochemical characteristics, antigenicity, and biocompatibility of collagen from the starfish, *A.*

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Abbreviations: BCIP/NBT, 5-bromo, 4-chloro, 3-indolylphosphate/nitro blue tetrazolium chloride; bFGF, basic fibroblast growth factor; DMEM/F12, Dulbecco's modified eagle medium: nutrient mixture F-12; EGF, epidermal growth factor; ELISA, Enzyme-linked immunosorbent assay; FBS, fetal bovine serum; HDF, human dermal fibroblast; PMS, N-methyl dibenzopyrazine methyl sulfate; PSC, pepsin solubilized collagen; SDS-PAGE, SDS-polyacrylamide gel electrophoresis; XTT, sodium 3'-[1-(phenylaminocarbonyl)-3,4-tetrazolium]-bis(4-methoxy-6-nitro)benzene sulfonic acid hydrate

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amurensis, and its potential for use with human dermal fibroblasts.

Materials and Methods

Starfish. Specimens of the starfish, *A. amurensis,* were collected along the coast of Yeosu, Jeonnam, Korea. Their tissues were excised, washed with distilled water, cut into small pieces, and stored at –25°C until used.

Preparation of collagen from starfish tissue. All preparative procedures were performed at 4°C. To remove the noncollagenous proteins and pigments, the tissues were extracted with 0.2 M $Ca(OH)_2$ over a period of 2 days and washed with distilled water.

The small pieces of tissue were suspended in 0.5 M acetic acid and digested with 10% (w/v) pepsin (EC 3.4.23.1; Showa, Tokyo, Japan) for 2 days. The viscous solution was centrifuged at 5,000 rpm for 30 min, and the supernatant was dialyzed against 0.02 M Na_2HPO_4 (pH 7.2) over a period of 3 days. The precipitate was dissolved in 0.5 M acetic acid and salted out by adding NaCl. The resultant precipitate was dissolved in 0.5 M acetic acid, dialyzed against 0.1 M acetic acid, and then lyophilized, forming pepsin solubilized collagen (PSC).

SDS-polyacrylamide gel electrophoresis (SDS-PAGE). SDS-PAGE was performed on a gradient separating gel of 5% polyacrylamide using 3.75% stacking gel according to Laemmli's method [Laemmli, 1970], but with slight modifications. After electrophoresis, each gel was stained with Coomassie Brilliant Blue R-250 (Sigma, St. Louis, MO) and destained with 25% methanol and 7.5% acetic acid.

CM-Toyopearl 650M column chromatography. Subunits of the starfish collagen were separated by using a CM-Toyopearl 650M (Tosoh Co., Tokyo, Japan) column chromatograph. The collagen sample (20 mg) was dissolved in 5 mL of 20 mM sodium acetate buffer (pH 4.8) containing 6 M urea at 4°C and denatured at 40°C for 30 min. The denatured collagen sample was chromatographed in the CM-Toyopearl 650M column (1.0×5.0 cm), which had been previously equilibrated with the same buffer, using a linear gradient of 0-0.2 M NaCl in the same buffer at a flow rate of 0.8 mL/min. Subunits were detected by measuring the UV absorbance at 230 nm.

Peptide mapping. Collagen samples were digested with lysyl endopeptidase from *Achromobacter lyticus* (EC. 3.4.21.50; 4.5 amidase activity/mg protein; Wako, Osaka, Japan) or glutamyl endopeptidase from *Staphylococcus aureus*, strain V8 (EC. 3.4.21.19, Sigma), at 37°C for 30 min. SDS-PAGE was performed by the method of Laemmli *et al*. [1970] using 15% gel or 12% gel, respectively.

Amino acid composition. Samples were hydrolyzed with 6 N HCl under vacuum at 110°C for 24 h, and the hydrolysates were analyzed on a LKB-4150α amino acid analyzer (LKB Biochrom. LTD, Cambridge, UK).

Denaturation temperature. Viscosity was determined with a viscometer (Model DV-III; Brookfield, Middleboro, MA) using spindle No. 1 and a speed of 100 rpm. The collagen solution was heated from 4°C to 50°C with a heating rate of 4°C/min. Measurements were carried out in triplicate.

Solubility. Collagen solubility was determined by the method of Montero *et al*. [1991] with a slight modification. The collagen was dissolved in 0.5 M acetic acid to obtain a final concentration of 3 mg/mL, and the mixture was stirred at 4°C until the collagen was completely solubilized. An aliquot of collagen solution (8 mL) was transferred to a centrifuge tube, and the pH was adjusted with either 6 N NaOH or 6 N HCl to a final pH range from 1 to 11. The solution was centrifuged at 5,000 rpm at 4°C for 30 min. Protein content in the supernatant was determined with a Bio-Rad Protein Assay Kit (500-0002, Bio-Rad, Hercules, CA) using bovine serum albumin as a standard. The effect of NaCl on collagen solubility was measured with 5 mL of collagen (6 mg/mL) in 0.5 M acetic acid. This sample was mixed with 5 mL of a series of concentrations of NaCl at 0, 2, 4, 6, 8, 10, and 12% (w/v) in 0.5 M acetic acid. The mixture was stirred at 4°C for 30 min, then centrifuged at 5,000 rpm at 4°C for 30 min. Protein content was measured as above.

Western blotting. After separation by SDS-PAGE, collagen chains were transferred to nitrocellulose membrane (Amersham, Piscataway, NJ) and then immunostained [Ramshaw and Werkmeister, 1988]. The nitrocellulose membrane was blocked with 5% bovine serum albumin solution and then reacted with primary antibody (polyclonal type I collagen antibody, ab292, Abcam, Cambridge, UK) for 1 h and with secondary antibody (Anti-rabbit IgG, Sigma, St. Louis, MO) for 30 min. The primary antibody was a dilution of 1:1000 and the secondary antibody was diluted 1:5000. The binding of antibodies was visualized with BCIP/NBT (Sigma).

Human dermal fibroblast cultures. Human dermal fibroblast (HDF) from foreskin was employed in this study. The HDF was isolated by enzymatic digestion of foreskin by 2 mg/mL collagenase from *Clostridium hystolyticum* (Sigma) overnight at 37°C. Isolated cells were subsequently cultured in DMEM/F12 (Gibco BRL, Gaithersburg, MD) supplemented with 10% FBS (Hyclone, Logan, UT), 20 μL/L bFGF (Sigma), 50 μL/L EGF (Sigma), 50 μg/L L-ascorbic acid (Sigma), and 1 IU/mL heparin (Sigma) in culture flasks. The culture was placed

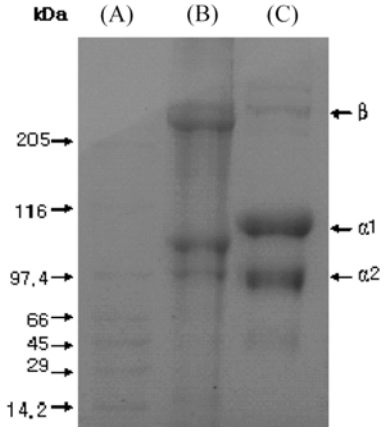

Fig. 1. SDS-PAGE of calf skin type I collagen and starfish collagen on 5% gel. (A), molecular weight marker; (B), calf skin collagen; (C), starfish collagen.

in an incubator (Sanyo, Osaka, Japan) at 37°C with a 5% CO_2 atmosphere; the media was changed every 2 days. HDFs from the third passage were used in the experiment.

XTT Assay. Cell growth activity in the collagen was tested with XTT (sodium 3'-[1-(phenylaminocarbonyl)-3,4-tetrazolium]-bis(4-methoxy-6-nitro)benzene sulfonic acid hydrate) labeling reagent and PMS (N-methyl dibenzopyrazine methyl sulfate) (Roche, Mannheim, Germany). PSC samples were gelated at 37°C and diluted from 0.01 mg/mL to 5 mg/mL. HDF cells in concentrations of 5.0×10^3 cells/well and 1.0×10^4 cells/well were placed in a 96-well plate for 4 h. Then, PSC samples were added to each well and incubated for 24 h. After 24 h, 50 mL of XTT labeling mixture was added and incubated for 24 h. XTT assay is based on the cleavage of the yellow tetrazolium salt XTT to form an orange formazan dye by metabolically active cells. During the incubation period, formazan products were measured at intervals of 4 h using an ELISA spectrophotometer reader (Bio-tek, NY, USA) at 490 nm with a reference wavelength of 690 nm. The absorbance was correlated to the cellular proliferation and activation.

Results and Discussion

The content of PSC produced by the present method was approximately 5.8% of the wet tissue. This result was different from that obtained by Kimura *et al.* [1993], who obtained a yield of about 5 g from a starting body-wall wet weight of 50 g. We examined the collagen with SDS-PAGE using 5% gel and found that it consisted of two different α bands, α1 and α2, and a β-component band. There was a clear 2:1 ratio between the α1 and α2 bands (Fig. 1). Thus, this structure is similar to type I collagen.

The collagen was further fractionated by CM-Toyopearl

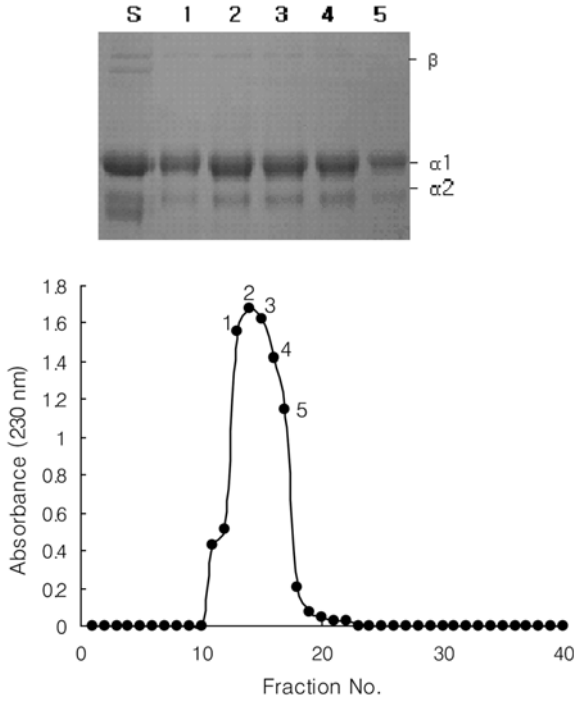

Fig. 2. CM-Toyopearl 650M column chromatography of denatured starfish collagen. The column (1.0×5.0 cm) was equilibrated with 20 mM sodium acetate buffer, pH 4.8, containing 6 M urea. Adsorbed proteins were eluted with a linear gradient of 0-0.2 M NaCl in same buffer at a flow rate 0.8 mL/min. The fractions indicated by the numbers 1-5 were examined by SDS-PAGE. (S) Loaded sample.

650M column chromatography and analyzed by SDS-PAGE. As indicated by the number (Fig. 2), the results suggested that the collagen consisted of two α chains. The collagen was revealed to be a heterotrimer with a chain composition of $(\alpha1)_2\ \alpha2$.

The amino acid composition of the PSC is shown in Table 1. The collagen was high in proline, glycine, and alanine, which is a characteristic of collagens in general [Ogawa *et al.*, 2004]. High levels of hydroxylysine and hydroxyproline, comparable to levels observed in collagens from mammalian species, were also found in the collagen. In addition, the collagen was similar to fibrillar collagen from the body wall of *A. amurensis,* as reported by Kimura *et al.* [1993], but distinct from other invertebrate collagens so far studied in that they contained a relatively high content of alanine and a relatively low content of hydroxylysine.

The denatured collagen was examined by V8 protease and lysyl endopeptidase digestion in order to compare it with calf skin collagen. The collagens produced similar, but not identical, peptide maps (Fig. 3). This result shows that the peptide fragments of this collagen were different from that of calf skin collagen.

Table 1. Amino acid composition of starfish collagen, residues/1,000

Amino acid	
Hydroxyproline	61
Aspartic acid	60
Threonine	24
Serine	72
Glutamic acid	83
Proline	97
Glycine	338
Alanine	107
Half-cysteine	0
Valine	25
Methionine	9
Isoleucine	18
Leucine	18
Tyrosine	4
Phenylalanine	5
Hydroxylysine	7
Lysine	15
Histidine	4
Arginine	53
Total	1,000

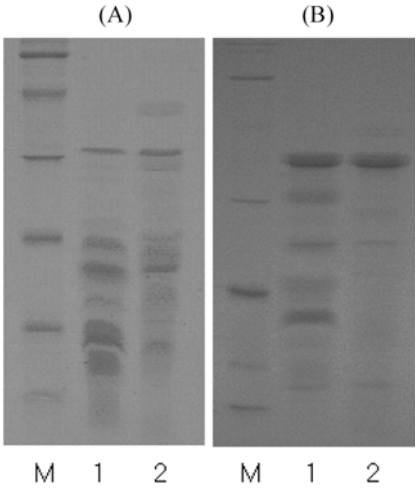

Fig. 3. Peptide maps of lysyl endopeptidase (A, 15% gel) and V8 protease (B, 12% gel) digests of calf skin collagen (1), starfish collagen (2), and marker (M).

The thermal denaturation curve of the starfish collagen sample is shown in Fig. 4. The denaturation temperature was 24.7°C, which was about 12°C lower than that of calf skin collagen, but about 2°C higher than that observed by Kimura *et al*. [1993]. This result demonstrates that the collagen's thermal stability correlates well with its low content of total amino acids [Kimura *et al*., 1993]. It is also known that the stability of collagen is correlated to environmental and body temperatures [Rigby, 1968].

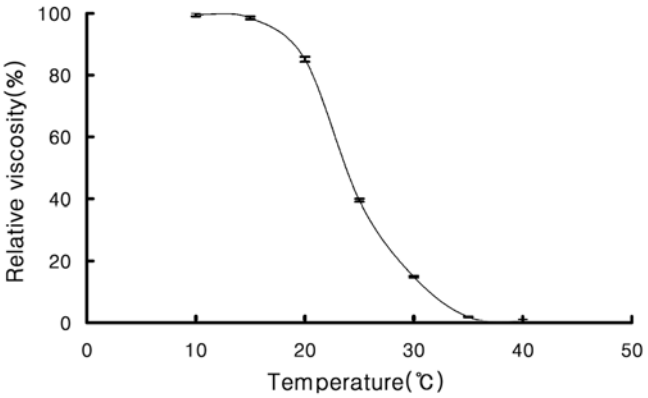

Fig. 4. Denaturation temperature of the solution of starfish collagen as measured by relative viscosity.

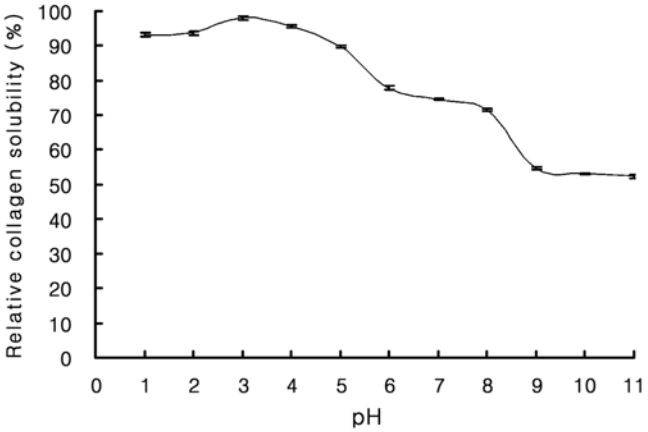

Fig. 5. Relative solubility of collagen from starfish at different pH levels.

The effects of pH and NaCl concentrations on starfish collagen solubilities are shown in Figs. 5 and 6, respectively. The solubility of collagen was found to be higher at pH 3.0 than at neutral pH. When pH values are above or below the isoelectric point, a protein has a net negative or positive charge, respectively. As a consequence, more water interacts with the charged protein [Vojdani, 1996]. Therefore, decreased solubility was found in the alkaline pH range. Charge repulsion contributes to greater protein solubility [Vojdani, 1996]. Starfish collagen showed high solubility at pH <6.0 (Fig. 5). Solubility of collagen was maintained at a high level in the presence of NaCl up to a concentration of 3% NaCl, then decreased with higher concentrations (Fig. 6). At a higher concentration, NaCl might result in decreased protein solubility (salting out effect) by increasing hydrophobic interaction and aggregation and competing with the protein for water, thereby causing the protein to precipitate [Kittiphattanabawon *et al*., 2005].

Under immunoblot assay, the constituent subunits of the type I collagen of starfish and calf skin showed very

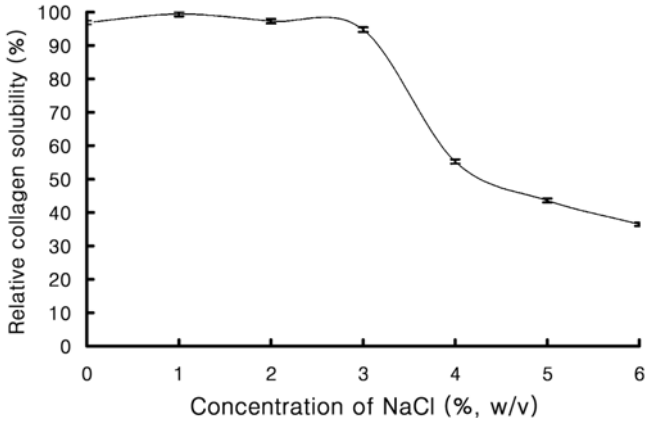

Fig. 6. Relative solubility in 0.5 M acetic acid of collagen from starfish in the presence of NaCl at different concentrations.

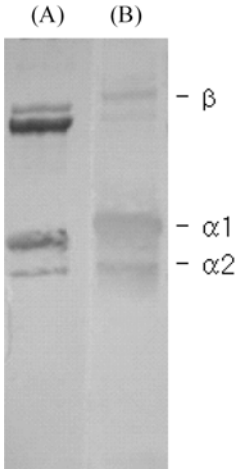

Fig. 7. Immunoblot analysis after SDS-PAGE and transference to nitrocellulose membrane and immunostaining. (A), calf skin collagen; (B), starfish collagen.

specific reactivity to the anti-type I collagen antibody. Fig. 7 shows the result of immunoblot analysis for type I collagen of starfish and calf skin. The immunoblot pattern of starfish was similar to that of calf skin collagen. The anti-type I collagen antibody was mainly reactive for the α1 and α2 chains. These results suggest that type I collagen is widely distributed in the tissue of starfish and defines the antigenicity of the PSC. The natural polymer of a telopeptide poor collagen like PSC is of low antigenicity, biocompatible, biodegradable, and less toxic than synthetic polymers consisting of a mixture of monomeric and oligomeric molecules when extracted from starfish tissues by pepsin digestion [Ho *et al*., 1997].

HDF isolated from foreskin was employed to measure the cytotoxicity of starfish collagen using the XTT assay. An increase of living cells resulted in an increase in the

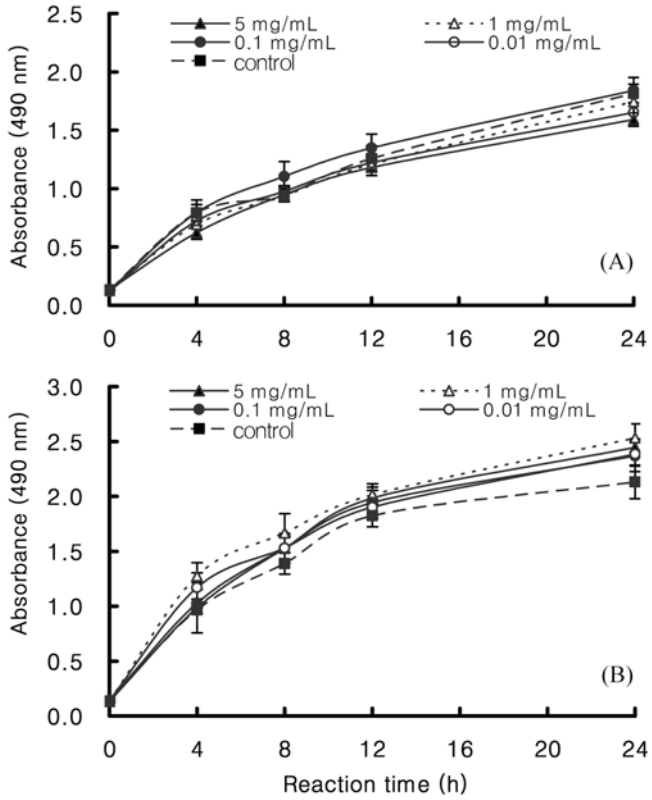

Fig. 8. HDF cell viability in starfish collagen by XTT assay. Effect of increasing PSC suspension concentrations for 0 h, 4 h, 8 h, 12 h, and 24 h. (A), 5×10^3 cells/well; (B), 1×10^4 cells/well.

overall activity of mitochondrial dehydrogenases in the sample. This increase directly correlates to the amount of orange formazan formed, as monitored by the absorbance. The results of this evaluation of mitochondrial activity *in situ* are shown in Fig. 8A and 8B. PSC induced the proliferation of HDF at a concentration of 5.0×10^3 cells/well and 1.0×10^4 cells/well. HDF cell survival was greater at 0.1 mg/mL than the control at 5.0×10^3 cells/well (Fig. 8A). At 1.0×10^4 cells/well, cell growth activity was still higher in HDF collagen concentrations than in the control (Fig. 8B). Therefore, PSC suspension was shown to encourage cell growth rather than cell toxicity.

In this study, we detected type I collagen in the tissue of starfish by biochemical and immunochemical techniques. Additionally, the collagen did not exhibit any biocompatibility problems with human dermal cells. However, further study to lower the collagen's denaturation temperature will be necessary to enable wide industrial application.

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