

## Physico-biochemical Characteristics of Scallop Mantle Collagen Soluble in Pepsin

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### ABSTRACT

Collagen, which is widely distributed in pluricellular animals, is one of the most fundamental constituents of the extracellular matrix, and plays mechanically or physiologically important roles in their bodies. In this study, the biochemical and physical characteristics of pepsin-solubilized collagen from the mantle of Yesso scallop (YMPC), a by-product of processing, was determined. Electrophoretic patterns showed that scallop mantle collagen contained  $\alpha 1$  and  $\alpha 2$  chains, which was similar to the patterns of bovine tendon type V collagen. The YMPC showed different profiles in molecular, amino acids, peptide maps from those of bovine tendon collagen and lower denaturation temperature. Electron microscopic view of YMPC showed a sponge-like structure in part. These results indicated that YMPC may become a sustainable source of useful collagens for various purposes including value-added biomaterials. It may also be useful in a variety of applications as an alternative of vertebrate collagen, which has been widely used.

**Keywords:** By-product, Denaturation temperature, Mantle, Peptide map, Solubilization.

### INTRODUCTION

Collagen is the most abundant animal protein representing nearly 30% of total protein in the body. It is a major protein of connective tissues such as tendon, skin, bone, the vascular system of animals, and the connective tissue sheaths surrounding muscle (Foegeding *et al.*, 1996). Since collagen is an important biomaterial, the amount and properties of the composed collagen in meat have become an important index for texture evaluation in the fields of food science and industry (Senaratne *et al.*, 2006). Skin and bone from bovine and porcine sources have usually been utilized in collagen production. However, the out-break of the mad cow disease (BSE) in the 1980s accelerated the search for a collagen alternative. Another motivation for finding an alternative to

mammalian collagen is that Muslims, Jews, and Hindus do not accept collagen produced from bovine and/or porcine sources. Many scientists have found that skin, bone, fin and scales of both fresh water and marine fishes, chicken skin, marine sponge, and bull frog skin can be used as alternatives, which have no ethnic- or safety-related consumer concerns (Sadowska *et al.*, 2003; Nagai *et al.*, 2000, 2001; Muyonga *et al.*, 2004a; Swatschek *et al.*, 2002; Nam *et al.*, 2008; Gom'ez-Guill'en *et al.*, 2011). On the other hand, invertebrate collagen, which occupies 95% of the whole animal collagen, was inferior to vertebrate one (Elijah, 1978).

Scallop is a cold water shellfish aquacultured mainly in Korea and Japan. Scallop mantle is consistently by-produced more than 30,000 tons per year in Japan and Korea, which may become a sustainable

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source of useful collagen. In a previous study (Nam *et al.*, 2008), the squid skin collagen and its enzymatic hydrolysate was characterized for the utilization as an ingredient in food and cosmetic industry. Therefore, the objective of this study was to investigate the biochemical and physical characteristics of scallop mantle collagen such as denaturation degree, electron micrograph, peptide mapping, electrophoresis, and amino acid composition, etc., for utilization in food, pharmaceutical, and cosmetics industries.

## MATERIALS AND METHODS

Live scallop, *Patinopecten yessoensis*, was purchased in June, 2009 at a local fish market Joomoonjin, Korea, and stored at -40°C until used. Bovine tendon collagen was purchased from Sigma Chemical Co. (St. Louis, MO, USA). All other reagents used were of analytical grade.

### Preparation of Scallop Mantle Collagen

All operations were done below 4°C. Scallop mantles (500 g) were homogenized with 5 volumes (v/w) of distilled water. The homogenate was centrifuged at 10,000×g for 20 minutes. To remove the non-collagenous proteins, 20 volumes (v/w) of 0.1N NaOH was added. The suspension was stirred overnight and then centrifuged three times at 10,000×g for 20 minutes. The precipitate was washed thoroughly with distilled water. The precipitate was then suspended in 10 volumes (v/w) of 0.5M acetic acid and solubilized by limited digestion with porcine pepsin (P6887, Sigma Chemical Co., St. Louis, MO, USA) at a substrate: enzyme weight ratio of 20:1 for 2 days. The suspension was centrifuged at 10,000×g for 1 hour, and the supernatant was adjusted to 0.7 M NaCl concentration by adding 4.0M NaCl to precipitate the solubilized collagen. The precipitate was collected by centrifuging at 5,000×g for 20 minutes, re-dissolved in 0.5M acetic acid, and then

centrifuged at 30,000×g for 1 hour. Finally, the supernatant was dialyzed against distilled water and then lyophilized.

### Amino Acid Composition

For determination of amino acid composition, the collagen was hydrolyzed with 6N HCl at 110°C for 24 hours. The hydrolysate was analyzed with an automatic amino acid analyzer (Hitachi L-8800, Tokyo, Japan). The amino acid content was expressed by the number of residues per 1,000 residues.

SDS (Sodium Dodecyl Sulphate)-PAGE (Polyacrylamide Gel Electrophoresis)

SDS-PAGE was performed according to the method of Laemmli (1970) using 7.5% separation and 5% stacking gels. Two mg of the extracted collagen was added in 1 mL of sample buffer containing 2-mercaptoethanol until a final concentration of 2 mg mL<sup>-1</sup> of sample was reached, as described by Gómez-Guillén *et al.* (1997). Approximately 20 µL of sample solution was loaded onto sample wells and electrophoresed. Gels were stained with 0.1% Coomassie Brilliant Blue R-250 and then destained with 10% methanol and 10% acetic acid. Molecular marker (S8320, Sigma Chemical Co., St. Louis, MO, USA) consisted of myosin, 205 kDa; β-galactosidase, 116 kDa; phosphorylase B, 97.4 kDa; bovine serum albumin, 66 kDa; glutamic dehydrogenase, 55 kDa; ovalbumin, 44 kDa; glyceraldehyde-3-phosphate dehydrogenase, 36 kDa.

### Peptide Mapping

Peptide mapping was performed by hydrolyzing the collagen with glutamyl endopeptidase of *Staphylococcus aureus* strain V8 (EC 3.4.21.19, Sigma Chemical Co., St. Louis, MO, USA). One mg of sample was dissolved in 0.5M phosphate buffer (pH 7.2) containing 0.5% SDS and boiled for 2 minutes. After adding 0.5 mL of

the same buffer, 15  $\mu\text{g}$  of V8 protease was added to the collagen solution and incubated at 37°C for 25 minutes. Hydrolysis was quenched by boiling for 3 minutes. SDS-PAGE of the proteolysis was performed as mentioned above using 10% separation and 5% stacking gels.

### Denaturation Temperature

The collagen (2-3 mg) was suspended in 10  $\mu\text{L}$  of distilled water and the solution was applied to a differential scanning calorimeter (DSC 2910, TA Instruments, New Castle, DE, USA). All thermograms were recorded from 10 to 50°C at a constant heating rate of 0.5  $^{\circ}\text{C min}^{-1}$ . The denaturation temperature of collagen was determined by using software provided by the manufacturer.

### Electron Microscopy

Sample was coated with platinum for 5 minutes in the holder of Field Emission Scanning Electron Microscopy (FE-SEM S-4700, Hitachi, Japan). Then, the coated sample was observed using FE-SEM.

### Statistical Analysis

Statistical analysis of the data was carried out by Duncan's multiple comparison test ( $P \leq 0.05$ ) using the SPSS software package version 10.0 program from SPSS Inc. (Chicago, IL, USA).

## RESULTS AND DISCUSSION

### Amino Acid Composition

Although some differences in amino acid composition were apparent across collagens derived from different sources, the composition of collagen encompasses all 20 amino acids (Schrieber and Gareis, 2007).

The amino acid compositions of scallop mantle and bovine tendon collagens are shown in Table 1. Based on the results, glycine, as the most abundant amino acid in all collagens, followed by glutamic acid occupied approximately 23 and 14%, respectively. There were also relatively high contents of proline (9%), hydroxyproline (8%), alanine (4%), arginine (7%), and aspartic acid (9%) in scallop mantle collagen. These results were in line with previous work by Shen *et al.* (2007). They mentioned that glycine and glutamic acid were highest (33.1 and 11.1%, respectively) in the amino acid composition of scallop harvested in Hokkaido, Japan. However, there were some differences in amino acid contents between our sample and those obtained in Japan, which can be related to environmental conditions, latitude, and feeding patterns. The proline and hydroxyproline contents are approximately 30% for mammalian gelatins, 22–25% for warm-water fish gelatins (Tilapia and Nile perch), and 17% for cold-water fish gelatin (cod) (Muyonga *et al.*, 2004b). Balian and Bowes (1977) reported that only mammalian protein contained large amounts of hydroxyproline and hydroxylysine, hence the total imino acid (proline and hydroxyproline) content of scallop collagen was relatively high. Scallop mantle collagen showed lower imino acid content than bovine tendon collagen (Table 1). Hydroxyproline is believed to play a singular role in the stabilization of the triple-stranded collagen helix due to its hydrogen bonding ability through its  $-\text{OH}$  group (Burjandze, 1979; Ledward, 1986). Johnston-Banks (1990) reported that the imino acids imparted considerable rigidity to the collagen structure and that a relatively limited imino acid content should result in a less sterically hindered.

### SDS-PAGE Pattern

The molecular weight distribution is also an important and meaningful parameter for

**Table 1.** The amino acid compositions of scallop mantle collagen\*.

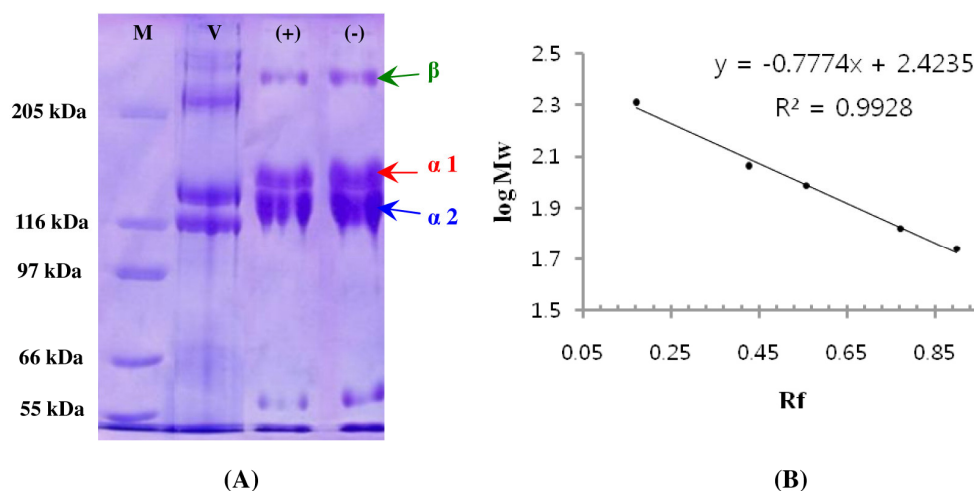
Amino acid	Collagen		
	Scallop mantle collagen	Bovine tendon collagen type I	Bovine tendon collagen type V
Asp	97	62	59
Thr	32	21	19
Ser	52	34	33
Glu	137	107	93
Gly	233	223	229
Ala	44	88	88
Val	20	28	26
Met	27	10	10
Ile	26	18	16
Leu	41	36	35
Tyr	17	11	11
Phe	21	24	25
Lys	10	35	32
His	7	8	8
Arg	77	82	85
Pro	89	112	115
Hyp	84	101	101
Hyls	16	0	16
Imino acids (Pro+Hyp)	173	213	216
Total	1000	1000	1000

\*Residues per 1.000 residues.

assessing the technological properties of collagen. The molecular weight distribution is used in the selection of special types of collagen for particular applications or for obtaining certain functional properties by blending different types of collagen or molecular weight fractions. SDS-PAGE patterns of scallop mantle and bovine tendon collagens are shown in Figure 1-A. Scallop mantle collagen had at least two different  $\alpha$  chains ( $\alpha 1$  and  $\alpha 2$ ) and one  $\beta$ -chain. According to SDS-page results, similar patterns obtained for 2-mercaptoethanol-treated and untreated scallop mantle collagens, which are related to non-existence of disulfide linkages in their chain. Therefore, it can be concluded that molecular species of type III and IV collagen are not involved in the extracted collagen (Shen *et al.*, 2007). The existence of at least two different subunits ( $\alpha 1$  and  $\alpha 2$ ) demonstrates that the major scallop mantle collagen is type V collagen. In addition, the thicker SDS-PAGE bands of scallop mantle

collagens implied less homogeneity of polypeptide chains. This could cause unstable triple-helical collagen structures with lower denaturation temperature.

The electrophoretic positions of  $\alpha$  chains of scallop mantle collagen were different from those of bovine tendon collagen in respect of the molar mass of polypeptide chains (Figure 1-A). The  $\alpha 1$  and  $\alpha 2$  chains of scallop mantle collagen had higher molecular weight than those of bovine tendon collagen. Both scallop mantle and bovine tendon collagens contained intra- and intermolecular cross-linked component,  $\beta$  (dimer) chain. These kinds of dimer were also observed in the collagens originated from marine vertebrates and invertebrates (Nam *et al.*, 2008; Morales *et al.*, 2000; Lin *et al.*, 2005; Hwang *et al.*, 2007). Shen *et al.* (2007), on the other hand, reported that the digested scallop mantle collagen showed an electrophoretic pattern similar to our electrophoretic pattern, which was heterotrimer chain.



**Figure 1.** (A) SDS-PAGE patterns of scallop mantle collagen with (+) or without (-) 2-mercaptoethanol. M: Molecular markers; V: Bovine tendon type V collagen, (B) Standard curve for determination of the molecular weight. Log Mw: Llog 10 of the molecular weight, Rf: Relative mobility value.

The molecular weight of each subunit was calculated according to the following equation:

$\log Mw = -0.7774 Rf + 2.4235$  (Figure 1-B). The molecular weights of  $\beta$ ,  $\alpha 1$ , and  $\alpha 2$  were 227, 147, and 130 KDa, respectively.

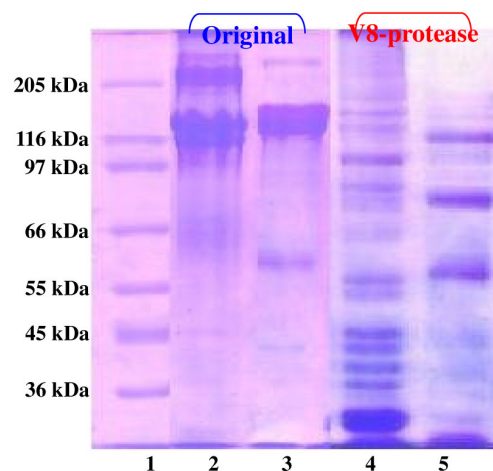
### Peptide Mapping

To compare the primary structure of scallop mantle and bovine tendon collagens, enzymatic hydrolysis with V8 protease was carried out. The resulting peptides were mapped by SDS-PAGE gel (Figure 2). The two collagens showed different mapping patterns, which means that both primary structures were different from each other. There were substantial decreases in the band intensities of cross-linked chain ( $\alpha$  chains) in peptide maps of scallop mantle collagen digested by V8 protease, which resulted in lower molecular weight fragments; however, most chains appeared to be intact from V8 protease attack. The chains of bovine tendon collagen were hydrolyzed to more extent. This result suggested that  $\alpha$  chains as well as their cross-link chains of scallop mantle collagen were more resistant to digestion by

V8 protease than those of bovine tendon collagen.

### Denaturation Temperature

The denaturation temperatures ( $T_d$ ) of scallop mantle and bovine tendon collagens were investigated using a differential scanning calorimeter in order to evaluate the thermal stability.  $T_d$  of scallop mantle collagen was 27.4°C, while that of bovine tendon collagen was 37.0°C (Table 2). Moreover, the collagen extracted from scallop mantle in Japan (Mizuta *et al.*, 2007) showed higher  $T_d$  (30-35°C) compared to our sample, which may correlated with their body and environmental temperatures where they are living (Rigby, 1968; Pati *et al.*, 2010). Invertebrate collagen indicated similarity with  $T_d$  of squid skin collagen (Nam *et al.*, 2008), in which the collagen with higher transition temperature had greater stability in high-temperature environments. Aquatic collagen also had lower  $T_d$  than collagen of land mammals (Nagai *et al.*, 2001; Lin *et al.*, 2005; Senaratne *et al.*, 2006), which was in a good agreement with this study. Moreover,  $T_d$  of



**Figure 2.** Peptide mapping of scallop mantle collagen hydrolyzed by V8 protease. Lane 1: Molecular markers; Lane 2: Bovine tendon type V collagen; Lane 3: Scallop mantle collagen; Lane 4: Hydrolyzed bovine tendon type V collagen, Lane 5: Hydrolyzed scallop mantle collagen.

**Table 2.** Denaturation temperature of scallop mantle collagen.

Collagen	Denaturation temperature (°C)
Scallop mantle	27.4 <sup>b</sup>
Bovine tendon	37.0 <sup>a</sup>

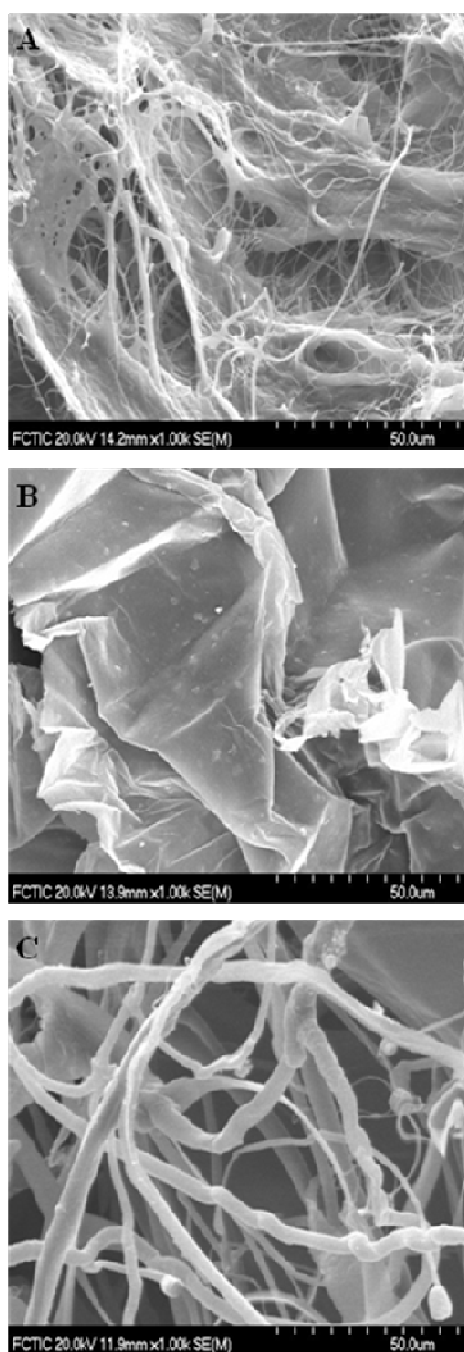
<sup>a, b</sup> Means in the same column with different superscripts are significantly different ( $P < 0.05$ ).

collagen from warm water fish is higher than that of cold water fish (Takahashi and Yokoyama, 1954). In general, fish collagens have lower imino acid (proline and hydroxyproline) contents than mammalian collagens, which may be the reason for denaturation at low temperature (Grossman and Bergman, 1992; Pati *et al.*, 2010). Scallop mantle collagen had a lower content of Ala than bovine tendon one. This amino acid, together with Pro and Hyp, is found in nonpolar regions where sequences of the type Gly-Pro-Y predominate (Ledward, 1986). A higher content of these amino acids, that is, Pro, Hyp, and Ala, in commercial collagen from bovine tendon is one of the major causes responsible for its higher denaturation temperature properties (Ledward, 1986).

It was also reported that Td of collagen was proportionally correlated with the imino acid content, proline, and hydroxyproline. The imino acids were suggested to affect the thermal stability of tri-polypeptide helical structures (Nomura *et al.*, 2000; Nagai *et al.*, 2000; Li *et al.*, 2004; Liu *et al.*, 2005). These reports were almost the same as this study, in which the imino acid contents of scallop mantle collagens (17.3%) were significantly lower than that of bovine tendon type V collagen (21.6%).

### Electron Microscopy

The appearance and bulk structure of bovine tendon type V and scallop mantle collagens were observed with a scanning electron microscopy (Figure 3). Bovine tendon type V collagen was observed to have a complex fibril form. As a consequence, bovine tendon collagen (A in Figure 3) will have high wettability. Scallop mantle crude collagen (B in Figure 3) only treated with NaOH appeared to be a plane-sheet-like film with a dense structure. On the other hand, the purified collagen of scallop mantle (C in Figure 3) showed fibrils structure similar to bovine tendon type V collagen, which was thicker in scallop



**Figure 3.** Scanning electron micrograph of collagens. (A) Bovine tendon type V; (B) Crude scallop mantle collagen, (C) The purified scallop mantle collagen.

mantle collagen than bovine tendon. Cross-linked fiber networks may be mediated by hydrogen bond, hydrophobic interaction, electrostatic bond, and entropic and dispersions forces (Nemethy *et al.*, 1963).

## CONCLUSIONS

The SDS-page results showed similar patterns obtained for 2-mercaptoethanol-treated and untreated scallop mantle collagens, which are related to non-existence of disulfide linkages in their chain. Based on this reason, molecular species of type III and IV collagen would not appear in the scallop mantle collagen. The existence of at least two different subunits ( $\alpha 1$  and  $\alpha 2$ ) were evidence that the major scallop mantle collagen was type V.

Scallop mantle collagen had lower imino acid content and Td than bovine tendon collagen. Collagen extracted from scallop mantle showed different compositional and physicochemical properties from bovine tendon collagen. Moreover, the differences between amino acid contents and Td of collagen obtained in our work with those extracted from scallop mantle in Japan showed that environmental conditions, latitude, and feeding patterns could be affective parameters on final properties of the scallop mantle collagen. Lower denaturation temperature of scallop mantle collagen implied possible utilization in different industries. Further studies are now in progress to investigate the application of enzymatic hydrolyzed scallop collagen in food, pharmaceutical, and cosmetic industries.

## ACKNOWLEDGEMENTS

This study was supported by the Regional Core Research Program funded by the Korea Ministry of Education, Science and Technology (Medical and Bio-material Research Center), and the grant (No. RTI05-01-02) of the Regional Technology Innovation Program of the Ministry of Knowledge Economy (MKE), Korea. Kyoung Gook Lee and Sabnam are the recipient of a graduate fellowship provided by the Brain Korea (BK21) program sponsored by the Ministry of Education, Science and Technology, Republic of Korea.



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## ویژگیهای فیزیکوزیست شیمیایی کولاژن تن پوش (جبه) صدف خوراکی (اسکالوپ) محلول در پپسین

ج. ه. چویی، ش. بهنام، و س. ک. کیم

### چکیده

کولاژن که به طور گسترده ای در حیوانات پرسلولی وجود دارد یکی از اساسی ترین مواد تشکیل دهنده ماتریکس برون یاخته ای است و نقش های مکانیکی یا فیزیولوژیکی مهمی را در اندام آنها بر عهده دارد. در این مطالعه، ویژگیهای فیزیکوشیمیایی کولاژن محلول در پپسین گرفته شده از جبه اسکالوپ یسکو (Yessco) (با مخفف YMPC) که محصول جانبی فرآوری است، تعیین شد. نقشبندی های الکتروفورز نشان داد که کولاژن جبه اسکالوپ با داشتن زنجیره های  $\alpha 1$  و  $\alpha 2$  مشابه نقشبندی تیپ V زردپی گاوی است. YMPC تفاوتی را از نظر پروفیل مولوکولی، اسیدهای آمینه، و نقشه های پپتیدی با کالوژن زردپی گاوی نشان داد و نیز دارای درجه حرارت واسرشتی کمتری بود. تصویر میکروسکوپ الکترونی YMPC در بخشهایی ساختاری اسفنجی را نشان میداد. این نتایج حاکی از این بود که YMPC ممکن است منبع پایداری از کالوژن مفید برای مصارف گوناگون از قبیل مواد



زیستی با ارزش اضافی باشد. همچنین ممکن است این ماده کالوژن مهره داران را که به طور گسترده برای کاربردهای متنوع استفاده می شوند جایگزین کند.