Isolation and Characterization of Pepsin-Soluble Collagen from Abalone (*Haliotis discus hannai*) Gastropod Muscle Part II

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Abalone (*Haliotis discus hannai*) gastropod muscle was divided into Part I (the center part of gastropod muscle) and Part II (the transforming and virginal part of gastropod muscle). Pepsin-soluble collagen was extracted from abalone gastropod muscle part II (PSCAGM-II) with yield of 8.7%. SDS-PAGE and amino acid composition indicated that the PSCAGM-II contained α₁ and α₂ chains and might be classified as type I collagen. PSCAGM-II exhibited a maximum absorbance at 232 nm, but little absorbance near to 280 nm. FTIR investigations showed the existence of helical arrangements of collagen. PSCAGM-II triple helical stereochemical structure was destroyed when the treated temperature up to 35°C for 10 min. Denatured temperature detected by the viscometer was 22.9°C when incubated for 20 min. Study on the properties and thermal stability of PSC from abalone gastropod muscle has certain directive significance for the abalone processing industry.

Keywords: abalone (*Haliotis discus hannai*), gastropod muscle, pepsin-soluble collagen, characterization

Introduction

Abalone is known as one of the most expensive marine food products because of its delicious taste and rich nutrition, especially in Asian countries (Olley and Thrower, 1977). In recent years, abalone aquaculture production grows rapidly in China. Gastropod muscle is the major edible organ of abalone and is mainly composed of collagen (and myofibril?). Myofibrils are surrounded by very thick layers of collagen fibrils of about 1 mm, which show cross striation patterns with a typical periodicity of about 60 nm (Olaechea et al., 1993). Although gastropod muscles are very solid, they have high degrees of morphological complexity and behavioral plasticity (Voltzow, 1990). This composition and structure of abalone gastropod muscle made it difficult to process in food industry. Gastropod muscle can be divided into center part (Part A or Part I), transforming part (Part B) and marginal part (Part C) by its morphous (Fig. 1). In the present study, Part B and Part C were together named Part II for study. Previous study had suggested that the collagen content ratio of Part II and Part I was about 10 to 1 (Kimura and Kubota, 1968). It meant that the transforming and marginal part (Part II) of abalone gastropod muscle contained much more collagen comparing to the central part (Part I).

![Fig. 1. Illustration of sampling locations of abalone *Haliotis discus hannai*.](image-url)
Collagen is the main component of extracellular matrix (ECM) and has gained application in various fields for its unique physiochemical property and excellent biocompatibility. Collagen molecules are structural macromolecules included in their structure one or several domains that have a characteristic triple helical conformation (Rest and Garrone, 1991). It is the major fraction of connective tissue and has been used in food, pharmaceutical and photographic industries (Zhou and Regenstein, 2006). The study on collagen properties of echinoderm previously had been reported in sea cucumber (Saito et al., 2002), sea urchin (Omura et al., 1996) and sea stars (Kimura et al., 1993). Most of collagen from these species are type I collagen and they possessed a relative stability.

Since abalone muscle is easily hardening and browning, there are lots of difficulties in abalone processing in the food industry. In abalone gastropod muscle part II, collagen content is relatively high. A positive correlation was found between the texture change and collagen properties, including relative content, fibril structure, and gelatination (Olaechea et al., 1993; Hatae et al., 1996; Gao et al., 2001). Reports showed that there was a positive correlation between abalone gastropod muscle tenderness and collagen content (Olaechea et al., 1993; Hatae et al., 1996). Furthermore, toughness is an important index of abalone processing product and it affects the mouth feeling of products with different processing and storage conditions. Therefore, it could be thought that biological features of gastropod muscles are likely to be at least partly related to the molecular properties of their abundant collagens.

In view of the Asian propensity for abalone consumption, it is necessary to study the character of the abalone collagen to benefit for abalone processing and storage. Therefore, study on characteristics of collagen from abalone gastropod muscle part II will provide meaningful guidance for the abalone processing and storage. Recent years, lots of papers focused on the practical utilization of marine animals to produce collagen (Matmaroh et al., 2011; Pallela et al., 2011; Zhang et al., 2009a; Wang et al., 2008). In the present study, the objective was to evaluate biochemical properties and thermal stability of collagen from abalone muscle part II, and to correlate these properties with the structure of collagen molecule. Since abalone collagen content was distinct with seasonal change and discrepancy in different part of abalone organ (Hatae et al., 1995), the abalones in April were collected for study.

**Materials and Methods**

**Sample preparation** Abalone (*Haliotis discus hannai*, the weight is 75 ± 5 g, the size is about 7.5 × 5 cm) was collected in April in Dalian, Liaoning Province. After removing the shell of fresh abalone, gastropod muscle part II (65 ± 5% in the whole) were taken for collagen extraction.

**Extraction of collagen** The extraction was according to procedures described by Chie Tanaka-Yoneda with some modify (Tanaka-Yoneda et al., 1997). All the preparations were carried out at 4°C. Briefly, the abalone part II of gastropod muscle was separated from abalone, homogenated and washed by 10 volumes of deionized cold water. The precipitate was collected (centrifugation, 5000 × g, 10 min) and stirred with 10 volumes of 0.6 M KCl for 24 h. After centrifuged at 5000 × g for 15 min, the precipitate was treated with the above procedure for two more times. Subsequently, the precipitation was dissolved in 10 volumes of 0.45 M NaCl stirring for 24 h. After centrifugation at 5000 × g for 15 min, the precipitate was treated with the same procedure for two more times. The precipitate was then performed with 10 volumes of 0.1 M NaOH stirring for 12 h to remove non-collagen materials. The extract was washed by deionized water to neutral pH then crude collagen was collected.

The crude collagen was suspended with 10 volumes of 0.5 M acetic acid agitating for 48 h to remove the acid-soluble collagen. After centrifugation at 10000 × g for 30 min, the insoluble fraction was digested with pepsin in pH 2.0, 4°C for 72 h in 0.5 M acetic acid circumstances. The ratio of enzyme and substrate was 3/20. The product was centrifuged at 12000 × g for 10 min and the PSCAGM-II in the supernatant was salted out by the addition of NaCl to a final concentration of 0.8 M. The precipitate was collected (12000 × g, 10 min), dissolved in 0.5 M acetic acid, dialyzed with 0.02 M Na₂HPO₄ and 0.1 M acetic acid for 2 d to remove salt, and finally lyophilized and weight for analysis.

**UV-vis spectra** The Ultraviolet absorption spectra of PSC was recorded by LAMBDA 35 spectrophotometer (Perkin Elmer Inc, USA) from 190 to 400 nm at a scanning rate of 210 nm/min. The PSCAGM-II was dissolved in 0.5 M acetic acid to obtain a concentration of 0.1% (m/v).

**Fourier transform infrared spectroscopy (FTIR)** FTIR spectra of PSCAGM-II was obtained from tablet containing 2 mg collagen with 200 mg potassium bromide (KBr). Spectra was recorded using a FTIR spectrophotometer (Perkin Elmer Inc, USA) of Spectrum One-B from 4000 to 400 cm⁻¹ at a data acquisition rate of 0.5 cm⁻¹ per point.

**Amino acid analysis** The collagen dry powder was hydrolysis by 6 M HCl at 110°C for 24 h (Yan et al., 2009). The hydrolysate was derived with O-phthalaldehyde (OPA) and stored in dark. After cooling to room temperature and filtration with 0.45 μm microporous membrane filter, the product was applied to high performance liquid chromatography (HP1100, USA) equipped with a reversed-phase
column and detector of HPLC-DAD for amino acid composition analysis (Fürst et al., 1990). The PSCAGM-II typical peptide segment was measured by MS/MS (Q-TOF2, Micromass, Britain) with electrospray ion-source at the Mass Spectrometry Laboratory of National Center of Biomedical Analysis (Beijing, China).

**SDS-polyacrylamide gel electrophoresis (SDS-PAGE)**

SDS-PAGE was performed according to the method of Laemmli (Laemmli, 1970) using 8% separation gel and 5% stacking gel. 20 μL collagen solution was mixed with 5 μL sample buffer 5 × (60 mM Tris-HCl, pH 6.8 containing 14.4 mM β-mercaptoethanol, 2% SDS, 25% glycerol, 0.1% bromophenol blue). Sample was heated at 100°C for 5 min and centrifuged at 5000 g for 10 min to remove undissolved debris. After electrophoresis, the gel was stained with 0.25% (w/v) Coomassie Brilliant Blue (CCB) R250 and destained with mixed solution of water, methanol and acetic acid (3.5/3/2, v/v/v). High-molecular-weight markers (Takara Co., Japan) were used to estimate the molecular weights of the collagen.

**Circular dichroism (CD) spectra**  Four portions of 0.02% (m/v) PSCAGM-II in 20 mM acetic acid solution were heated at 20°C, 35°C, 60°C, 90°C for 10 min, respectively. The blank sample was without heating. The thermal treated samples were injected to colorimetric cylinder and analyzed by circular dichroism spectra (Jasco J-810, Japan). Each colorimetric cylinder was scanned from 190 nm to 250 nm with speed of 100 nm/min to accumulate for 3 times.

**Relative viscosity**  PSCAGM-II (0.32‰, m/v) in 0.1 M acetic acid was subjected to ubbelohde viscometer. PSCAGM-II solution was heated from 15°C – 40°C with a heating rate of 2.5°C/min. The temperature point with specific viscosity of the sample changed for 50% was defined as denaturing temperature (T_d). After the PSC solution was heated at different temperature for 20 min, the samples were measured by Ubbelohde viscometer. The relative viscosity was calculated in comparison to that obtained at 4°C.

**Results and Discussion**

**Extraction of PSC from abalone gastropod muscle**  The abalone gastropod muscle was hardly solubilized in 0.5 M acetic acid. Therefore, the residues were re-extracted by pepsin with the substrate weight ratio of 15%. The yield of pepsin-soluble collagen from abalone gastropod muscle part II (PSCAGM-II) was 8.7% (on the dry basis). This result was much lower than those of paper nautilus (50%) (Nagai, et al., 2002), ocellate puffer (44.7%) (Nagai, et al., 2002) and grass carp skin 46.6% (Zhang et al., 2007) and was higher than that of sea cucumber 2.3% (Saito et al., 2002) by acid extraction. The different yields of ASC and PSC of those marine animal species suggest that there were many intrachain cross-links at the telopeptide region of the collagen that resulted in low solubility in acid. After adding pepsin, the cross-linked regions at the telopeptide were cleaved without damaging the integrity of the triple helix, leading to the high solubility of collagen in acid (Zhang et al., 2007). Collagen from abalone gastropod muscle part II might contain too many intrachain cross-links at the telopeptide region, which resulted in an acid unsoluble phenomenon. Cross-links increase the mechanical and thermal stability of collagen fibers as well as their tensile strength, and this is correlated with the toughness of meat (Bailey, 1984). Previously, Jeremiah et al. (2003) and Riley et al. (2005) reported that insoluble collagen is an important factor in meat tenderness. Therefore, we supposed that PSCAGM-II contained intrachain cross-links partly related to the toughness of abalone muscle.

**SDS-PAGE pattern of PSCAGM-II**  The PSCAGM-II was examined by SDS-PAGE using an 8% gel. Lane A was the molecular weight markers (B) PSCAGM-II. Fig. 2. SDS-polyacrylamide gel electrophoresis of PSCAGM-II on 8% gel. (A) molecular weight markers (B) PSCAGM-II.
triple helical, $\beta$ was the dimer and $\gamma$ was the trimer structure. Therefore it could be speculated that PSCAGM-II was composed of three $\alpha$ chains. And this speculation was in agreement with previous reports of Tanaka-Yoneda (1997) and Kimura (1974) but was distinct from the report of Yoneda (1999). Basing the mRNA extraction, the abalone collagen had been proved to have two type $\alpha$ chains (Yoneda et al., 2000).

Make reference point to the existing of trace band, our presumption can be adjusted that there were two $\alpha$ chains, the obviously $\alpha$ chain ($\alpha_1$) and the trace $\alpha$ chain ($\alpha_2$) co-consisted the triple helix collagen. The trace $\alpha$ chain can be attributed to the material different, our samples were taken from the part II of abalone gastropod muscle (Fig.1). The different abalone habitats may contain different types of collagen. Most probably that the material contained two different types of collagen, one is the $\alpha_1$ chain trimer collagen, the other is collagen with two $\alpha_1$ chains and one $\alpha_2$ chain. And the type of $\alpha_2$ chain trimer collagen was dominant in the composition. That may be the reason that $\alpha_2$ chain was so ambiguous and it must be performed by immunoblotting for recognizing in SDS-PAGE (Yoneda et al., 2000). Therefore the PSCAGM-II was supposed to be mainly belong to the Collagen I chains for its mainly $\alpha_1$ chains trimer composition.

**Amino acid composition and sequence**  
Amino acid composition of PSCAGM-II was shown in Table 1. Glycine was the most abundant amino acid in PSCAGM-II with the content of 29.9%. This content is similar to the content of calf skin collagen (Girand-Guide, et al., 2000). Higher content of proline, hydroxyproline, alanine and glutamic acid but lower content of valine, cystine threonine, isoleucine and lysine were found in PSCAGM-II. The content is similar to that of the collagen from the body wall of sea cucumber (Satio et al., 2002). In contrast to low content of histidine and methionine in calf skin collagen amino acids, these amino acids were not detected in PSCAGM-II.

The imino acid (proline and hydroxyproline) content of PSCAGM-II was 22.1%, which was similar to those of pig skin (22%) and calf skin collagen (21.5%) (Ikoma et al., 2003; Cui et al., 2007), but was higher than those of several fish skin collagens (16 − 18%) (Gilsenan et al., 2000). The higher content of the imino acid, the more stable of the helices of collagen (Yan et al., 2009), because the molecular structure of collagen is maintained mainly by restrictions on changes in the secondary structure of the polypeptide chain, imposed by the pyrrolidine rings of proline and hydroxyproline, and also maintained partly by the hydrogen bond ability through the hydroxyl group of hydroxyproline (Zhang et al., 2007; Gustavson, 1995; Piez and Gross, 1960). Therefore, the PSCAGM-II helices might be as stable as mammalian skins, due to the similar imino acid content. The higher imino acid content and the formed stable helices could contribute to a better thermal stability and meat toughness (Smith and Judge, 1991). To be consistent with their results, our amino acid composition indicated that PSCAGM-II contained a higher level of imino acid. And the imino acids were contributed to the thermal stability of helices and greatly impacted meat quality including toughness of meat (Lawrie, 2006).

The amino acid sequence of a typical peptide segment of a chain from the PSCAGM-II was: RAGAPGPPGSQGVEGSPGSPG, showing the characteristic glycine-proline-Y and glycine-X-Y triplets of collagen. Six random peptide segments were obtained after the PSC was hydrolyzed by trypsin and the molecular weight was all below 4 kDa. The molecular weight and amino acid sequence was shown in Table 2.

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results was accordance with the sequence of Yoneda deduced from the cDNA clones (Yoneda et al., 2000). Our detected sequence GPSGPGSGEVQGPPGPAGAR was in agreement with the sequence deduced from α1 [AB017600] of GPSGPGSGEVQAGPPGPAGAR. And we thought the Ser most probably derived from Ala hydroxylation. Sequence of fragment No.4 and No.6 were a little different from cDNA sequence of α1 [AB017600] PGLPGQEGK and α2 [AB017601] VGPSGLPGLQGPAGPSGPGVEAGV, respectively. The reason might be attributed to the gene mutation or modification after translation. It was identified that the PSCAGM-II existed two type α chains. Moreover, there was no similar sequence of cDNA array matching that of fragment No.5. It could also be inferred that there was another α chain beside two type α chains which have been identified.

UV-vis spectra  Fig. 3 shows UV-Vis spectra of abalone collagens were scanned at wave-lengths of 190-590 nm. It was observed that the top peak was at 232 nm and there was a swell distributed in 250-290 nm. (Fig. 3)

It is generally considered that most proteins existed absorb light at 280 nm of UV-Vis spectra was aroused by tyrosine, tryptophan and phenylalanine. Since tryptophan did not exit in the PSC of abalone, tyrosine and phenylalanine content were 0.8% and 0.7%, the absorption peak at 280 nm was weak correspondingly. The top peak at 232 nm was similar to the skin collagen of largefin longbarbel (Zhang et al., 2009 b) and collagen of seaweed pipefish (Sher et al., 2009) at 233nm. This result indicated that the extract possessed a characteristic UV absorption of purified collagen.

Fourier transform infrared spectroscopy (FTIR)  Fig. 4 represents the Fourier transform infrared spectroscopy (FTIR) of the PSCAGM-II. There are some peak arrange from 3434 cm\(^{-1}\) to 639 cm\(^{-1}\) on the spectrum of graph, the main peak were found at 3434 cm\(^{-1}\), 2927 cm\(^{-1}\), 1648 cm\(^{-1}\) and 1554 cm\(^{-1}\).

The amide A band position was found at 3434 cm\(^{-1}\), which is the absorption band of N-H-stretching and shows that there were hydrogen bonds present. In the FTIR spectra the amide A band was relative to N-H stretching vibrations. The amide B was found at 2927 cm\(^{-1}\), it was contributing to the stretching of CH\(_2\). The spectra of PSCAGM-II disper -
sions also demonstrated the characteristic pattern reflecting the amid I band at 1554 cm\(^{-1}\), the amide II band at 1545 cm\(^{-1}\), and amide III band at 1237 cm\(^{-1}\), resulting from C=O-stretching, N-H bending vibrations and C-H-stretching (Payne and Veis, 1988), respectively. The amide I band which is associated with the secondary structure of the protein and the am-

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**Fig. 3.** Ultraviolet spectra of PSCAGM-II.

**Fig. 4.** FTIR spectra of PSCAGM-II.
ide III band demonstrated the existence of helical structure of the protein (Muyonga et al., 2004a, 2004b). Thus, the FTIR investigations suggest the existence of helical arrangements of PSCAGM-II.

**Thermal stability of PSCAGM-II** The heat transformation of PSCAGM-II is interpreted as disintegration of the collagen triple helical structure into random coils. This is accompanied by a change in physical properties, such as viscosity, sedimentation, diffusion, light scattering and optical activity (Usha and Ramasami, 2004). So firstly, we investigated the triple helical structure changes using circular dichroism.

The circular dichroism (CD) spectra of PSCAGM-II treated at various temperatures were shown in Fig. 5. The thermal treated PSCAGM-II showed a rotary maximum at 221 nm and minimum at 199 nm. Their consistent cross was over point at about 212 nm (Fig. 5).

The characteristic CD spectra curve described the typical triple helical conformation (Ikoma et al., 2003). It can be observed that the curve 1 and curve 2 were conformity and kept normal. When the treated temperature increased to 35°C or above, the CD spectra curves were severe changed. The positive peak was vanished, the negative peak became weaker, and the peak forms shifted a little. These phenomena indicated that the PSCAGM-II triple helical stereochemical structure was destroyed when the treated temperature up to 35°C above for 10 min.

The triple helical structure changed temperature is similar to that of pig skin (37°C) (Nagai et al., 2002). This result can explain why abalone is difficult to process or cook. Compared to the transition temperatures of the collagen from other species, such as bigeye snapper (28.7°C) (Kittiphathanabawon et al., 2005) and Brownstripe red snapper (30.5°C) (Jongjareonrak et al., 2005), the triple helical destroyed temperature of PSCAGM-II was a little higher.

Viscosity measurements are usually used to investigate the thermal stability of collagen. Fig. 6 showed the changes in fractional viscosity with increasing temperature and denaturation temperature (T_d) was calculated to be 22.9°C. Relative viscosity of PSCAGM-II change demonstrated that the higher temperature can denature the collagen. When reach at 22.9°C for 20 min, collagen hydrogen bonds were broken down and the collagen was denatured from triple helical structure into random coils (Wong, 1989). Compared to the other species, the denaturing temperature of PSCAGM-II was lower than that of skin and bone of bigeye snapper (Kittiphathanabawon et al., 2005, 2010). But it was similar to the denaturing temperature of Japanese sea-bass skins, chub mackerel, bullhead shark and ocellate puffer fish ranged from 25°C to 28°C (Kittiphathanabawon et al., 2005, 2010).

To combine the CD spectra and the relative viscosity data, we can draw the result that it was different between the triple helical destroyed temperature and denaturing temperature. This maybe due to the different treated method and time. However, the results indicated that the destroyed structure of PSCAGM-II needed a relatively high temperature or a long-time treatment.

Abalone is difficult to process or cook because it is easily hardening and browning. Our results indicated that PSCAGM-II contained too many intrachain cross-links at the telopeptide, similar amino acid composition and stable helices to calf skin and possessed relatively high destroyed

![Fig. 5. CD spectrum of PSCAGM-II. Curve 2, 3, 4, 5 was the CD signal of PSC, which was incubated at 20°C, 35°C, 60°C, 90°C for 10 min. Curve 1 was the signal of raw PSC.](image)

![Fig. 6. Thermal denatured curve of PSCAGM-II. The denatured temperature was measured by Ubbelohde viscometer in 0.5 M acetic acid. When specific viscosity changed for 50% the temperature point was defined to T_d. The thermal treatment temperature was distributed from 15−40°C (2.5°C interval) each temperature was incubated for 20 min.](image)
temperature comparing to other ocean animals. These results were consistent with a previous report that the content, solubility and thermal denaturing temperature of collagen were related to beef tenderness (Liu et al., 1995). Therefore, to a certain degree, our results explained well why abalone is difficult to process or cook as calf skin.

**Conclusion**

PSC extracted from abalone muscle part II with a yield of 8.7% (on the dry basis) and contained two α chains, α₁ and α₂. PSC exhibited a maximum absorbance at 232 nm, but little absorbance near to 280 nm. FTIR investigations showed the existence of helical arrangements of collagen. PSCAGM-II triple helical stereochemical structure was destroyed when the treated temperature up to 35°C for 10 min. Denaturation temperature detected by the viscometer was 22.9°C when incubated for 20 min. The collagen had a similar imino acid content to mammalian collagen and a better thermal stability.

**Acknowledgment**

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**Reference**


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