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Partial Purification of Mussel Adhesive Protein from *Mytilus Edulis* and Preparation of Mussel Protein Hydrolysates

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Abstract

Mussel adhesive protein (MAP) was extracted from Korean *Mytilus edulis* and then partially purified using Sephacryl S-300 gel permeation chromatography and reversed-phase high performance liquid chromatography. As an indicator of adhesiveness, its 3,4-dihydroxyphenylalanine (DOPA) content was determined. Its DOPA/protein ratio of 0.19 was higher than those of other reports, indicating a good adhesive. The partially purified MAP was confirmed by acid-urea polyacrylamide gel electrophoresis using cetylpiridinium bromide as a cationic detergent. Sea mussel hydrolysates were prepared using three commercial proteases to provide value-added functional materials and their angiotensin converting enzyme (ACE) inhibitory activities were determined. Among hydrolysates of sea mussel, Protamex was the best and further purification would improve ACE inhibitory activity.

Key words: mussel adhesive protein, *Mytilus edulis*, protein hydrolysates, ACE inhibitor

INTRODUCTION

Sea mussels such as *Mytilus edulis* make a secure adhesive byssus that bonds tightly to wet surfaces without extraordinary preparative treatment (1,2). The chemical component responsible for adhesion is a polyphenolic protein that is produced by an exocrine gland in the foot of mussel (3). The adhesive substance in the mussel is known to be a 130 kDa, tandemly repetitive protein, which contains a high proportion of 3,4-dihydroxyphenylalanine (DOPA) (4). Evidence for repeated peptide sequences in the purified protein was obtained by tryptic digestion and its amino acid analysis (5). The protein consists largely of 75~80 tandemly repeated hexa-(Ala-Lys-Pro-Thr-(Thr/DOPA)-Lys or deca-peptides (Ala-Lys-Pro-Ser-(Tyr/DOPA)-Hyp-Hyp-Thr-DOPA-Lys) (6). DOPA serves two roles (7). The first one is to compete with water molecules for strong adsorption to surfaces. Second, DOPA contributes to adhesive cross-linking with lysine.

There have been studies on the isolation and characterization of mussel adhesive protein (MAP) (5-10). Since the polyphenolic adhesive protein is stable in the acidic range around pH 3.0, extraction of MAP should be done in 5% acetic acid solution (5). The potential utilization of the MAP is as an adhesive under water, a biomedical adhesive, an opthalmic adhesive, and as a water impervious coating agent (11). Although MAP is a high value-added adhesive, there are no reports available regarding the extraction of MAP from Korean sea mussels. Therefore, in this study, we extracted and partially purified the MAP from the Korean sea mussel, *Mytilus edulis* and determined the DOPA content of the protein. Also, we prepared and studied the mussel hydrolysates to obtain the ACE inhibitory peptides, which can be used as a food ingredient containing functional components.

MATERIALS AND METHODS

Materials

Fresh sea mussels were obtained in Yousu, Korea. Flavorzyme (Novo Co., Switzerland), Protease NP (Pacific Co., Korea), and Protamex (Novo Co., Switzerland) were used as commercial proteases. All the other chemicals used were of analytical grade.

Pretreatment of sea mussel

The feet of sea mussels were separated and frozen immediately using liquid nitrogen and kept in a deep freezer. Edible portions of sea mussels were also treated under the same condition and kept for preparation of sea mussel protein hydrolysates.

Analysis of chemical composition of sea mussel

The chemical composition of sea mussel was analyzed according to the AOAC method (12).

Isolation of polyphenolic protein

MAP from *Mytilus edulis* was purified according to the method of Waite et al. (5). The frozen feet of *Mytilus edulis* were suspended in 0.05 M Tris buffer, pH 7.5, containing 10 mM N-ethylmaleimide, 25 mM EDTA, 1 mM potassium cyanide, soybean trypsin inhibitor, and 1M NaCl and extracted using a homogenizer. The homogenates were centrifuged at 10,000×g for 20 min. After discarding the supernatants, the pellets were suspended in 5% acetic acid containing 10 mM β-mercatoethanol and 1 mM PMSF and homogenized again. This was again centrifuged at 10,000×g. The

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supernatants were then dialyzed against distilled water. This was again dialyzed against 0.1 M sodium borate buffer, pH 8.7, containing 0.1% Triton X-100 and 1 mM EDTA and followed by centrifugation at 10,000×g for 20 min. The supernatants were again dialyzed against 5% acetic acid. And 5.5% guanidine hydrochloride and 0.001% Triton X-100 as a final concentration were added and ultra-filtered under nitrogen using a PM-10 membrane (Amicon Co., MA, USA).

Gel permeation chromatography (GPC)

Sephacryl S-300 column (1.5 cm×120 cm) was used as gel permeation chromatography. The elution solvent was 5% acetic acid solution and the flow rate was 10 ml/hr.

High performance liquid chromatography (HPLC)

Reversed-phase HPLC was performed using a C_8 column with a linear gradient of acetonitrile and 0.1% trifluoroaceticacid (TFA) was used as a eluting solvent.

Acid-urea PAGE

Acetic acid-urea polyacrylamide gel electrophoresis was performed according to the method of Panyim and Chalkley (13) with 5% acrylamide, 5% acetic acid, and 8M urea, pH 2.7 (7). As a running buffer, 5% acetic acid was used. Also, 5% acetic acid solution containing 4 M urea, 20% sucrose solution, 20% β-mercaptoethanol, and 0.001% cetylpiridinium bromide (CPBr) was used as a sample buffer.

Protein assay

Protein concentration was determined by Bradford's method (14) using BSA as a standard.

DOPA content assay

The concentration of DOPA was determined according to the method of Waite and Benedict (15). As an acid reagent, 0.5 M HCl was used and 1.45 M sodium nitrate solution containing 0.41 M sodium molybdate was used as a nitrate reagent. Also, 1 M NaOH was used as an alkaline reagent. One hundred micro liter of sample was added into a test tube and the acid reagent was added up to 0.3 ml as a total volume. And 0.3 ml of nitrate reagent was added and rapidly followed by addition of 0.4 ml of the alkaline reagent. The absorbance was monitored at 500 nm to determine the DOPA content based on the standard curve (Fig. 1).

Preparation of mussel protein hydrolysates

Mussel proteins were hydrolyzed with 1% of Flavorzyme, Protease NP, and Protamex at 50°C for 6 h, respectively and the hydrolysates were filtered using a PM-10 membrane (Amicon Co., MA, USA).

Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE)

SDS-PAGE was performed according to the method of Laemmli (16).

TNBS assay

The concentration of peptide was determined according to the modified TNBS method (17,18).

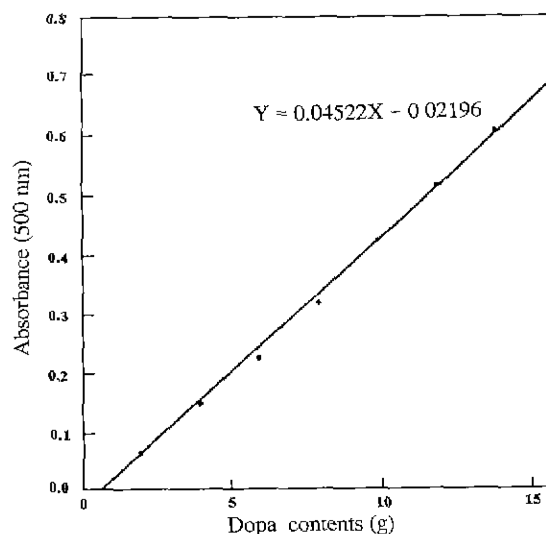

Fig. 1. Standard curve for determination of DOPA content.

Angiotensin converting enzyme (ACE) assay

ACE activity was measured by the modified method (19,20) of Cushman and Cheung (21). The reaction mixture contained 150 μl of 5 mM Hip-His-Leu as a substrate, 50 μl of rabbit lung ACE powder (5 m unit) in 50 mM sodium borate buffer (pH 8.3), and 50 μl of sample solution. The reaction was carried out at 37°C for 30 min, and terminated by the addition of 250 μl of 1 N HCl and 1 ml of ethylacetate. After centrifugation, the absorbance of the supernatant was measured at 228 nm.

RESULTS AND DISCUSSION

Chemical composition of sea mussel

The proximate composition of sea mussel is shown in Table 1. This result shows that sea mussel has about 8.9% protein which is the second largest component following water content. The protein content was a little less than the literature values (22,23). This difference is attributed to the harvest time and location of the sea mussels.

Partial purification of MAP from *Mytilus edulis*

Mussel adhesive protein from Korean *Mytilus edulis* was extracted from the feet of sea mussel. Since the polyphenolic adhesive protein is stable in the acidic range around pH 3.0, extraction was done in 5% acetic acid solution (5). The adhesiveness of the extracted MAP depends on the amount of DOPA. Therefore, the ratio of DOPA/protein is very impor-

Table 1. The chemical composition of sea mussel, *Mytilus edulis*

Composition	Content (%)
Moisture	79.38
Crude protein	8.90
Crude lipid	1.21
Carbohydrate	8.80
Ash	1.71

tant. Based on the standard curve (Fig. 1), the DOPA content of the extracted polyphenolic adhesive protein was determined (Table 2). The result shows that ultra-filtration increased the DOPA/protein ratio. Also, the decrease of the absolute amount of DOPA by ultra-filtration appears to be due to the oxidation of DOPA. The DOPA/protein ratio of 0.19 is higher than that of Waite et al. (1), indicating that the extracted polyphenolic protein is a very good adhesive. The extracted protein was further purified using GPC and reversed-phase HPLC. The elution profile of Sephacryl S-300 column chromatography is shown in Fig. 2. There were two major peaks eluted and the first peak (Peak 1), which contains the protein, was pooled and loaded onto the reversed-phase HPLC C_8 column to purify further. HPLC chromatogram (Fig. 3) showed a single peak at 12% of acetonitrile and it was confirmed on the acid-urea PAGE (Fig. 4). Since the polyphenolic protein is not soluble in an anionic detergent such as SDS, SDS-PAGE cannot be used. Therefore, acid-urea PAGE using CPBr as a cationic detergent was performed. Fig. 4 shows the typical pattern of the isolated polyphenolic protein with impurities as reported in the literature (4,5). It is assumed to have a molecular weight of 130 kDa. However, its yield was low and similar results were also reported in other studies (1,5). Therefore, more studies are needed to enhance the yield and characterize the MAP isolated from Korean *Mytilus edulis*.

Preparation of mussel protein hydrolysates

To find out the functional materials from mussel protein

Table 2. DOPA content of the polyphenolic adhesive protein extracted from sea mussel[1)]

	Protein (mg)	Dopa (mg)	Dopa/protein
Extracted	7.876	0.925	0.117
Ultra-filtered	3.335	0.634	0.190

[1)]About 30 g of sea mussel was used.

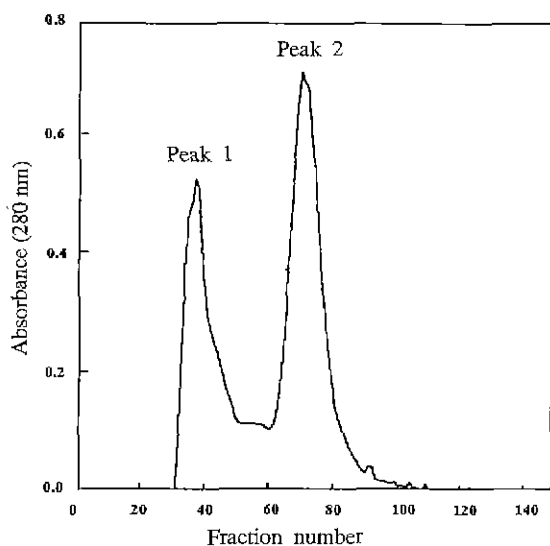

Fig. 2. Gel permeation chromatography elution profile of the mussel adhesive protein using Sephacryl S-300 column.

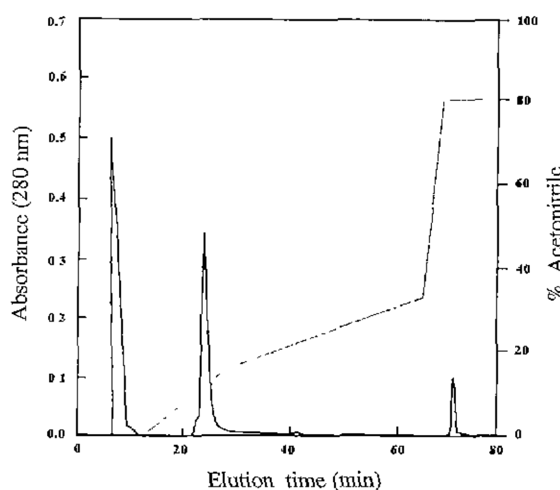

Fig. 3. Reversed-phase HPLC elution profile of the mussel adhesive protein.

Fig. 4. Acid-urea PAGE profile of the polyphenolic adhesive protein purified. Arrow indicates the partially purified MAP.

hydrolysates, mussel protein was enzymatically hydrolyzed using three different commercial proteases, Flavorzyme, Protease NP, and Protamex. The SDS-PAGE profiles of mussel protein hydrolysates are shown in Fig. 5. This result shows that sea mussel protein has 8, 15, 17, 28, and 50 kDa as major subunits. Hydrolysis of mussel protein with proteases produced smaller molecular weight molecules depending on the degree of hydrolysis. This result seems to support Lee et al.'s research (22), in which sea mussel was hydrolyzed for 4 h. For sea mussel protein, more than 4 h treatment with commercial proteases kept degrading the high molecular weight molecules into smaller ones.

The degree of hydrolysis of proteins and the available amino group concentrations of peptides can be measured by TNBS assay (17,18). Based on TNBS data, ACE inhibitory activities of mussel protein hydrolysates were measured and IC_{50} values

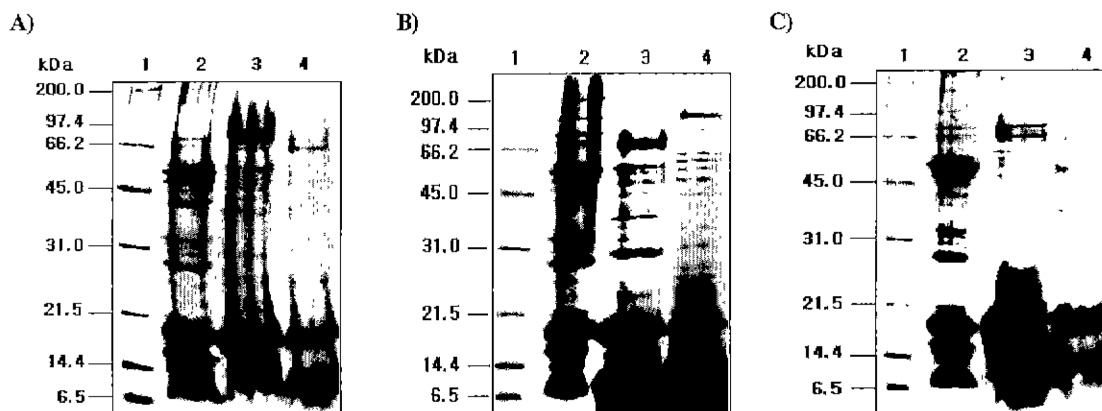

Fig. 5. SDS-PAGE profile of sea mussel protein hydrolysates using commercial proteases. A) Flavorzyme B) Protease NP C) Protamex Lane 1, Molecular weight marker; 2, 0 h hydrolysis; 3, 6 h hydrolysis; 4, 12 h hydrolysis

Table 3. ACE inhibitory activity of sea mussel protein hydrolysates[1)]

Enzyme	IC_{50} (mM)
Flavorzyme	5.20
Protease NP	5.90
Protamex	4.26

[1)]Six hour hydrolysis

(amount of chemical which causes 50% inhibition of enzyme activity) were determined, assuming that the available amino group concentration was directly related to peptide concentration. Among the enzymes used in this study, Protamex was the best in terms of IC_{50} values (Table 3). Although these IC_{50} values are relatively high, considering the crude fraction of ACE inhibitory materials, it is promising since more purification schemes would improve ACE inhibitory activity significantly. In conclusion, using the rather inexpensive raw materials of sea mussels, we report here that MAP can be obtained as a high value-added adhesive material and, also, crude preparation of enzymatic hydrolysates using commercial proteases could be a suitable way of producing ACE inhibitors.

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