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# A Novel Angiotensin I Converting Enzyme Inhibitory Peptide from Alaska Pollack (*Theragra chalcogramma*) Frame Protein Hydrolysate

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Alaska pollack frame protein, which is normally discarded as an industrial byproduct in the processing of fish in plants, was hydrolyzed with pepsin. This was fractionated into five major types of Alaska pollack frame protein hydrolysates (APH-I, 10−30 kDa; APH-II, 5−10 kDa; APH-III, 3−5 kDa; APH-IV, 1−3 kDa; and APH-V, below 1 kDa) using an ultrafiltration membrane bioreactor system. Angiotensin I converting enzyme (ACE) inhibitory activities of the fractionated hydrolysates were investigated, and the fraction that exhibited the highest ACE inhibitory activity was further purified using consecutive chromatographic methods on SP-Sephadex C-25 column, Sephadex G-25 column, and high-performance liquid chromatography (HPLC) on an octadecylsilane column. Finally, we purified a novel ACE inhibitory peptide with an $IC_{50}$ value of 14.7 $\mu$M, and the sequence of the peptide was Phe-Gly-Ala-Ser-Thr-Arg-Gly-Ala. In addition, the ACE inhibition pattern of the peptide was found to be noncompetitive.

**KEYWORDS: Alaska pollack frame protein; angiotensin I converting enzyme; noncompetitive**

## INTRODUCTION

Angiotensin I converting enzyme (ACE, EC 3. 4. 15. 1) plays an important physiological role in regulating blood pressure (*1*). ACE belongs to the class of zinc proteases and is located in the vascular endothelial lining of the lungs. It acts as an exopeptidase that cleaves dipeptides from the C terminus of various oligopeptides (*2*, *3*) and catalyzes the hydrolysis of angiotensin I to generate a potent vasoconstrictor, angiotensin II, and inactivates bradykinin, which has a depressor action.

Since the discovery of ACE inhibitors in snake venom, many studies have been attempted in the synthesis of ACE inhibitors such as captopril, enalapril, alacepril, and lisinopril, which are currently used in the treatment of essential hypertension and heart failure in humans (*4*, *5*). However, these synthetic drugs are believed to have certain side effects such as cough, taste disturbances, and skin rashes (*6*). Therefore, the search for natural ACE inhibitors as alternatives to synthetic ones is of great interest among researchers for safe and economical use.

Bioactive peptides can be released by enzymatic proteolysis of food proteins and may act as potential physiological modulators of metabolism during the intestinal digestion of the diet. The possible regulatory effects of peptides relate to nutrient uptake, immune defense, and opioid and antihypertensive activities. Recently, there have been many natural ACE inhibitory peptides isolated from the hydrolysis of various proteins such as cheese whey (*7*), casein (*8*−*10*), zein (*11*), tuna muscle (*12*), sardine (*13*), corn gluten (*14*), bovine skin gelatin (*15*), Alaska pollack skin gelatin (*16*), and bonito (*17*). These peptides are less potent than synthetic ones; however, they have not exhibited known side effects.

In the present study, we hydrolyzed Alaska pollack (*Theragra chalcogramma*) frame protein, which is normally discarded as an industrial byproduct in the processing of fish in plants, and purified a novel ACE inhibitory peptide. In addition, the sequence of the purified peptide and the inhibition pattern were also determined.

## MATERIALS AND METHODS

**Materials.** Alaska pollack frame was donated by Daerim Co. (Busan, Korea). ACE (from rabbit lung) and substrate peptide (hippuryl-histidyl-leucine) of ACE, SP-Sephadex C-25, and Sephadex G-25 were purchased from Sigma Chemical Co. (St. Louis, MO). Capcell Pak $C_{18}$-UG 120 (20 mm × 250 mm) and Capcell Pak $C_{18}$-UG 120 (10 mm × 250 mm) were purchased from Shiseido Co., Ltd. (Tokyo, Japan). All other reagents used in this study were reagent grade chemicals.

**Preparation of Pepsin Hydrolysate.** Alaska pollack frame protein was hydrolyzed with pepsin (3100 units/mg solid) as adjusting the substrate/enzyme ratio to 100:1 (w/w) at 37 °C for 8 h (pH 2.0). Alaska pollack frame protein hydrolysate (APH) was subsequently boiled for 10 min to inactivate the enzyme. The resultant APH was fractionated into five fractions (APH-I, 10−30 kDa; APH-II, 5−10 kDa; APH-III, 3−5 kDa; APH-IV, 1−3 kDa; and APH-V, below 1 kDa) using an ultrafiltration membrane (UF) system. All recovered APHs recovered were lyophilized in a freeze drier for 5 days.

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**Determination of ACE Inhibitory Activity of Fractionated APH.** The ACE inhibitory activity was measured by the method of Cushman and Cheung (*18*) with slight modifications. A sample solution (50 $\mu$L) with 50 $\mu$L of ACE solution (25 munits/mL) was preincubated at 37 °C for 10 min, and the mixture was incubated with 150 $\mu$L of substrate (8.3 mM Hip-His-Leu in 50 mM sodium borate buffer containing 0.5 M NaCl at pH 8.3) for 30 min at the same temperature. The reaction was terminated by the addition of 250 $\mu$L of 1.0 M HCl. The resulting hippuric acid was extracted with 0.5 mL of ethyl acetate. After centrifugation (800*g*, 15 min), 0.2 mL of the upper layer was transferred into a test tube and evaporated at room temperature for 2 h in a vacuum. The hippuric acid was dissolved in 1.0 mL of distilled water, and the absorbance was measured at 228 nm using an UV spectrophotometer (Cary 1C, Varian Inc., Australia). The $IC_{50}$ value was defined as the concentration of inhibitor required to inhibit 50% of the ACE activity.

**Purification of ACE Inhibitory Peptide.** The APH-V (7 g) was loaded onto a SP-Sephadex C-25 ion-exchange column (1.0 cm × 20 cm) equilibrated with 20 mM sodium acetate buffer (pH 4.0) and eluted with a linear gradient of NaCl (0−2 M) in the same buffer at a flow rate of 60 mL/h. The elution peaks were monitored at 280 nm, and the active fractions were pooled and lyophilized immediately. The lyophilized fraction was further purified on Sephadex G-25 gel filtration column (2.5 cm × 98 cm) equilibrated with distilled water. The column was eluted with distilled water, and 5.0 mL of fractions was collected at a flow rate of 60 mL/h. The fractions were detected at 280 nm. The fraction exhibiting ACE inhibitory activity was further purified using a reversed-phase high-performance liquid chromatography (RP-HPLC) on a Capcell Pak $C_{18}$ UG-120 (20 mm × 250 mm) column with a linear gradient of acetonitrile (0−70% in 50 min) containing 0.1% trifluoroacetic acid (TFA) at a flow rate of 4.0 mL/min. The elution peaks were detected at 215 nm, and the active peak was concentrated using a centrifugal evaporator. The peak representing the ACE inhibitory activity was rechromatographed on a Capcell Pak $C_{18}$ UG-120 (10 mm × 250 mm) column using a 15% acetonitrile containing 0.1% TFA at a flow rate of 2.0 mL/min.

**Determination of Amino Acid Sequence.** The sequence of ACE inhibitory peptide was determined by automated Edman degradation with a PerkinElmer 491 protein sequencer (Branchburg, NJ).

**Determination of the Inhibition Pattern on ACE.** Different concentrations of ACE inhibitory peptide were added to each reaction mixture according to Bush et al. (*19*) with some modifications. The enzyme activity was measured with different concentrations of the substrate. The kinetics of ACE in the presence of the inhibitor were determined by the Lineweaver−Burk plots.

## RESULTS AND DISCUSSION

**ACE Inhibitory Activity of Fractionated APH.** Every year, about 100 million tons of fish are harvested; however, 30% of the total catch is transformed into fishmeal (*20*, *21*). Over 50% of the harvest is a processing byproduct, which includes bone, skin, fins, internal organs, heads, and so on (*22*). In particular, fish frames obtained after filleting include bones, heads, and tails. The Alaska pollack frame contains approximately 61% of protein, which can be used as potential bioactive substances. Therefore, we investigated the ACE inhibitory activity of the enzymatic hydrolysate from Alaska pollack frame protein. Alaska pollack frame protein was hydrolyzed with pepsin under optimal conditions, and we fractionated five portions using an UF membranes bioreactor system having a range of molecular mass cutoffs (MWCO), i.e., 30, 10, 5, 3, and 1 kDa. The ACE inhibitory activity of fractionated APH is shown in **Table 1**. APH-V having a molecular mass (MW) below 1 kDa showed the highest ACE inhibitory activity. Therefore, APH-V was selected for further study.

**Isolation of ACE Inhibitory Peptide.** APH-V was dissolved in sodium acetate buffer (pH 4.0), loaded onto a SP-Sephadex C-25 column with the linear gradient of NaCl (0−2.0 M), and fractionated into five portions (**Figure 1**). Fraction IV was found to possess a strong activity with an $IC_{50}$ value of 0.11 mg/mL. Active fraction IV was subjected to size exclusion chromatography on Sephadex G-25 and fractionated into four portions (**Figure 2**). The fractions were pooled and lyophilized, and fraction IVB exhibited the strongest ACE inhibitory activity with an $IC_{50}$ value of 66 $\mu$g/mL. This active fraction was further separated by RP-HPLC on a Capcell Pak $C_{18}$ UG-120 (20 mm × 250 mm) using the linear gradient of acetonitrile (0−70%) containing 0.1% TFA, and the fractions were divided into six portions (**Figure 3**). Fraction FII showed the most potent ACE inhibitory activity with an $IC_{50}$ value of 23 $\mu$g/mL. To obtain a purified peptide, we rechromatographed on a Capcell Pak $C_{18}$ UG-120 (10 mm × 250 mm) using a 15% acetonitrile concentration containing 0.1% TFA (**Figure 4**). Finally, we

**Table 1.** ACE Inhibitory Activity of Fractionated APH by UF Membrane Bioreactor System

| hydrolysates | ACE inhibitory activity (%) |
|---|---|
| APH-V (MW 1 kDa below) | 87.62 |
| APH-IV (MW 1–3 kDa) | 78.95 |
| APH-III (MW 3–5 kDa) | 73.84 |
| APH-II (MW 5–10 kDa) | 68.24 |
| APH-I (MW 10–30 kDa) | 59.60 |

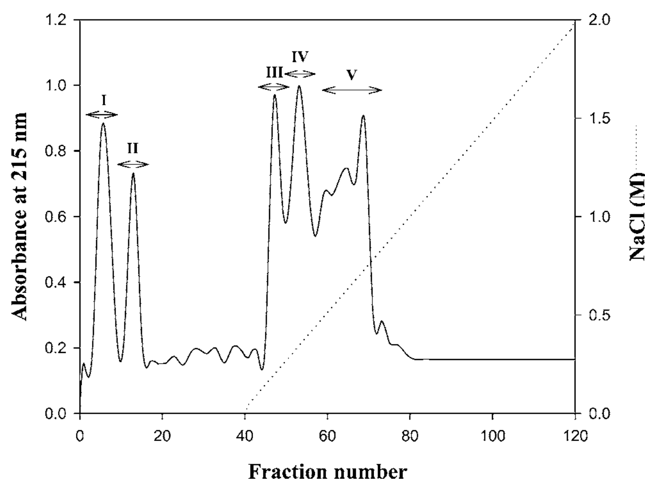


**Figure 1.** SP-Sephadex C-25 ion exchange chromatogram of APH-V. Elution was performed with the linear gradient of NaCl (0–2.0 M) at a flow rate of 60 mL/h. Protein elution was monitored at 280 nm.

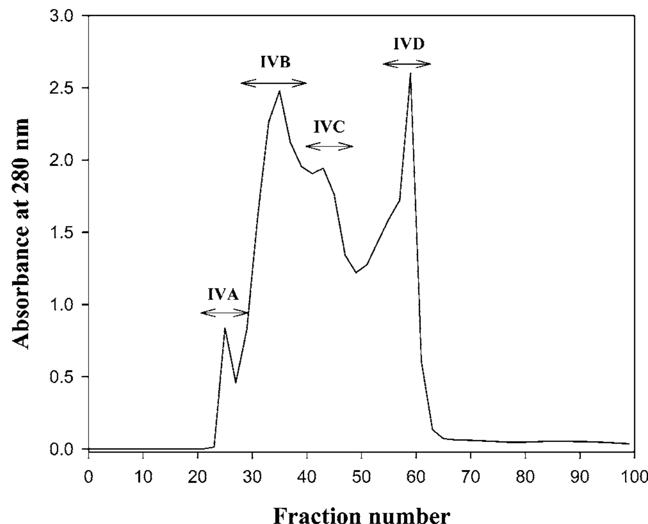


**Figure 2.** Elution profile of active fraction IV on a Sephadex G-25 gel filtration column.

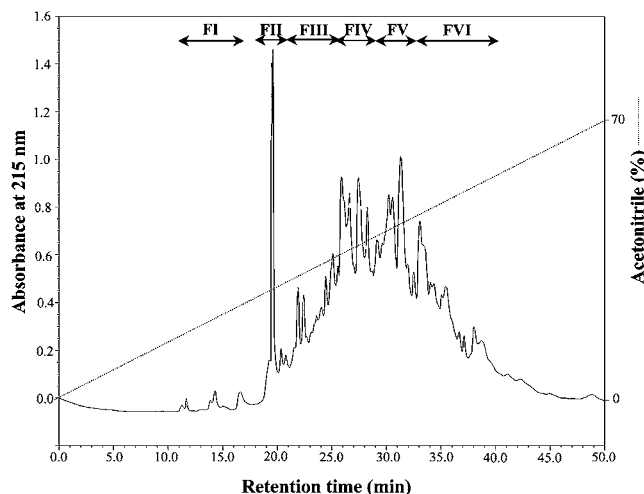


**Figure 3.** RP-HPLC $C_{18}$ chromatography of active fraction IVB obtained by gel filtration. Elution was performed with the linear gradient of acetonitrile (0–70%) containing 0.1% TFA.

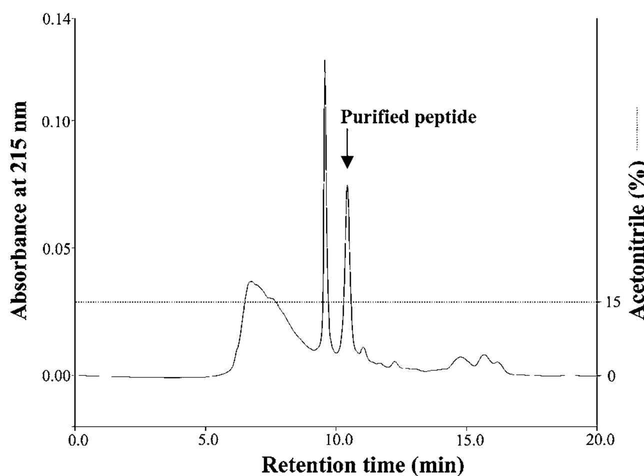


**Figure 4.** RP-HPLC $C_{18}$ rechromatography of active fraction FII. The elution was carried out with 15% acetonitrile containing 0.1% TFA.

**Table 2.** Purification of ACE Inhibitory Peptide from APH-V

| purification step | $IC_{50}$[a] (mg/mL) | yield (%) | purification fold |
|---|---|---|---|
| APH-V | 0.457 | 100 | 1.00 |
| SP-Sephadex C-25 | 0.110 | 33.57 | 4.15 |
| Sephadex G-25 | 0.066 | 8.39 | 6.92 |
| RP-HPLC | 0.023 | 0.09 | 19.87 |
| RP-HPLC | 0.013 | 0.03 | 35.15 |

[a] The $IC_{50}$ value was defined as the concentration of inhibitor required to inhibit 50% of the ACE activity.

obtained a purified peptide having an amino acid sequence as Phe-Gly-Ala-Ser-Thr-Arg-Gly-Ala with an $IC_{50}$ value of 14.7 $\mu$M. The results obtained during the purification steps are summarized in **Table 2**. The ACE inhibitory peptide was purified 35.15-fold from APH-V on a five-step purification procedure, and the yield was 0.03%.

**Determination of ACE Inhibition Pattern.** The ACE inhibition pattern of the peptide purified from APH-V was estimated using Lineweaver−Burk plots and was found to be noncompetitive (**Figure 5**). It means that the peptide can combine with an enzyme molecule to produce a dead-end complex, regardless of whether a substrate molecule is bound or not.

Recently, many ACE inhibitory peptides have been isolated from food proteins such as cheese whey (*7*), casein (*8*−*10*), zein (*11*), tuna muscle (*12*), sardine (*13*), corn gluten (*14*), bovine skin gelatin (*15*), Alaska pollack skin gelatin (*16*), and bonito (*17*). In addition, Phe-Phe-Val-Ala-Pro (*8*) and Ile-Pro-Pro (*9*) derived from casein exhibited an ACE inhibitory activity ($IC_{50}$ = 2.0 and 5.0 $\mu$M), and Leu-Gln-Pro (*11*) derived from zein showed an $IC_{50}$ value of 9.6 $\mu$M. Ile-Val-Gly-Arg-Pro-Arg-His-Glu-Glu ($IC_{50}$ = 6.2 $\mu$M), Ala-Leu-Pro-His-Ala ($IC_{50}$ = 10 $\mu$M), Phe-Gln-Pro ($IC_{50}$ = 12 $\mu$M), Leu-Lys-Pro-Asn-Met ($IC_{50}$ = 17 $\mu$M), Asp-Tyr-Gly-Leu-Tyr-Pro ($IC_{50}$ = 62 $\mu$M), and Ile-Lys-Pro-Leu-Asn-Tyr ($IC_{50}$ = 43 $\mu$M) were isolated from a thermolysin digest of dried bonito (*17*). In the present study, a novel ACE inhibitory peptide from APH-V was purified, and the peptide (Phe-Gly-Ala-Ser-Thr-Arg-Gly-Ala) were composed of hydrophobic amino acids at the C-terminal end with an $IC_{50}$ value of 14.7 $\mu$M. This $IC_{50}$ value exhibited a higher or similar activity as compared to those of peptides derived from dried bonito; however, it was a lower activity than those of peptides from the hydrolysate of casein and zein.

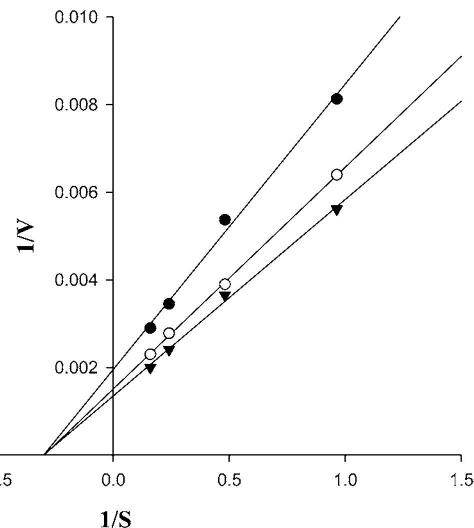


**Figure 5.** Lineweaver–Burk plots on ACE inhibitory activity in the presence of the peptide. Control (▼), 13 $\mu$g/mL of peptide (○), and 26 $\mu$g/mL of peptide (●).

Among the naturally occurring peptides with an ACE inhibitory activity, the most potent and specific inhibitors were several peptides with similar structures that have been isolated from the venom of the South American pit viper *Bothrops jararaca* and Japanese pit viper *Agkistrodon halys blomhoffi* (*23*, *24*). However, the structure−activity relationship of ACE inhibitory peptides has not yet been established because a large variety of peptides with different C-terminal amino acid sequence has been observed. ACE is strongly influenced by the C-terminal tripeptide sequence of the substrate, and it is suggested that peptides, which contain hydrophobic amino acids at these positions, are potent inhibitors. Furthermore, the positive charge as in the guanidine group of the C-terminal Arg contributes to the ACE inhibitory potency of several peptides, indicating that the binding site may be different from the catalytic site in ACE. In this study, a novel ACE inhibitory peptide from APH-V contained hydrophobic amino acids and Arg at the C-terminal tripeptide sequence, which may contribute to the ACE inhibitory activity.

In conclusion, we hydrolyzed Alaska pollack frame protein, fractionated five kinds of hydrolysate by an UF membrane bioreactor system, and purified five steps of consecutive chromatographic methods. The sequence of the purified peptide was Phe-Gly-Ala-Ser-Thr-Arg-Gly-Ala with an $IC_{50}$ value of

14.7 $\mu$M. In addition, the ACE inhibition pattern of the peptide was found to be noncompetitive.

## ABBREVIATIONS USED

ACE, angiotensin I converting enzyme; APH, Alaska pollack frame protein hydrolysate; MW, molecular mass; MWCO, molecular mass cutoffs; UF, ultrafiltration membrane; RP-HPLC, reversed-phase high-performance liquid chromatography.

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