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Purification of an ACE Inhibitory Peptide from Hydrolysates of Duck Meat Protein

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Abstract

An angiotensin converting enzyme (ACE) inhibitory peptide was isolated and purified from the hydrolysates of duck meat protein. Duck meat protein was hydrolyzed using trypsin at 37°C for 2 hrs. An ACE inhibitory peptide was purified using membrane filtration, anion exchange chromatography, gel permeation chromatography, fast protein liquid chromatography, normal phase HPLC. The purified inhibitory peptide was identified to be a tetrapeptide, Glu-Asp-Leu-Glu having IC_{50} value of 85.9 μM.

Key words: ACE inhibitor, duck protein, hydrolysates, isolation

INTRODUCTION

Bioactive peptides isolated from food proteins are potential modulators of various regulatory processes in the human body, and have the potential to reduce the risk of disease (1). Among biologically active peptides, angiotensin converting enzyme (ACE) inhibitory peptides have been extensively studied (2,3).

ACE converts angiotensin I into angiotensin II by cleaving the C-terminal dipeptide (His-Leu) of angiotensin I. ACE also inactivates bradykinin, a vasodiltor which depresses blood pressure. ACE inhibitors with the ability to decrease blood pressure have been isolated from various food sources (2). Since the discovery of an ACE inhibitory peptide in snake venom, many ACE inhibitory peptides have been identified from the enzymatic hydrolysates of various food sources. These include cheese whey (4), casein (5), zein (6), tuna (7), and soybean (8). However, there have been few studies on ACE inhibitors derived from meat proteins, although several antihypertensive peptides derived from fish and chicken muscle proteins have been reported (3,9).

Duck meat is considered to be a health food since it can reduce blood cholesterol levels (10). This study investigated duck meat protein as a functional food with cardiovascular benefits. Duck meat protein hydrolysate was prepared and a novel ACE inhibitory peptide was isolated and purified.

MATERIALS AND METHODS

Materials

Fresh duck (Aras platyrhynchos) meat was purchased in Daejeon, Korea. Duck meat was thoroughly washed using distilled water, separated into breast, wing, and thigh, and lyophilized. Lyophilized duck meat protein was used for enzymatic hydrolysis.

Preparation of duck meat protein hydrolysates

Duck meat protein was dissolved in 20 mM phosphate buffer (pH 7.5) and heated at 100°C for 10 min. The preheated sample was hydrolyzed using trypsin (porcine pancreas, EC 3.4.21.4, 14,900 unit/mg) with a 50:1 substrate to enzyme ratio (w/w) at 37°C for 0~5, or 6 hrs. Hydrolysis was terminated by heating the reaction mixture at 95°C for 10 min. Hydrolysates were then centrifuged at 6,300×g for 30 min.

Isolation of an ACE inhibitory peptide from hydrolysates

Duck meat protein hydrolysates were filtered using a YM-3 membrane (Millipore Co., Bedford, MA, USA). The membrane-filtered solution was loaded onto QAE-Sephadex (5×20 cm) equilibrated with 10 mM Tris buffer (pH 8.0) and eluted with a linear gradient of NaCl in the buffer from 0 to 0.5 M at a flow rate of 1 mL/min. The eluate was monitored by measuring the absorbance at 254 nm. The highest ACE inhibitory fraction from QAE-Sephadex was further purified using Sephadex G-15 (1.5×120 cm) equilibrated with 10 mM phosphate buffer (pH 7.0) at a flow rate of 0.3 mL/min. This eluate was monitored by measuring the absorbance at 214 nm. The highest ACE inhibitory fraction from Sephadex G-15 was further purified using FPLC (Amersham Pharmacia Co., Uppsala, Sweden) with a Resource Q column (6.4×30 mm, Amersham Pharmacia Co., Uppsala, Sweden) equilibrated with

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10 mM Tris buffer (pH 8.0) and eluted with a linear gradient of NaCl in the buffer from 0 to 0.5 M at a flow rate of 0.5 mL/min. Finally, to purify the ACE inhibitory peptide, the highest inhibitory fraction from Resource Q was loaded onto normal phase HPLC (Thermo Separation Products Inc., FL, USA) with an amino column (4.6×250 mm, Capcell pak NH2, Shiseido Co., Tokyo, Japan). Elution was performed on the condition of solvent A (0.1% trifluroacetic acid and 0.1% triethylamine in acetonirile: water (97 : 3, v/v) and solvent B (0.1% trifluroacetic acid and 0.1% triethylamine in acetonirile : water (30 : 70, v/v), having gradient of 0% of B to 80% at 0.5 mL/min.

TNBS assay

Peptide concentration was determined according to the trinitrobenzene sulfonic acid (TNBS) method (11) with a slight modification. TNBS (0.02 mL, 1.1 M) was added to 1 mL of the sample in sodium borate buffer (0.1 M, pH 9.2), mixed rapidly, held 5 min at room temperature and the reaction stopped by adding 2 mL of a freshly prepared sodium sulfite solution (1.5 mM). The absorbance was read at 420 nm against a blank.

Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE)

SDS-PAGE was performed according to the method of Laemmli (12). Protein samples for SDS-PAGE were prepared by mixing with sample buffer (60 mM Tris-HCl, 2% (v/v) SDS, 14.4 mM β-mercaptoethanol, 25% (v/v) glycerol, 0.1% (v/v) bromophenol blue, pH 6.8). Proteins were resolved on a 15% separation gel and stained with Coomassie Brilliant Blue. The following standard marker proteins purchased from Sigma Chemical Co. (St. Louis, MO, USA) were used: rabbit muscle myosin (205 kDa), *E. coli* β-galactosidase (116 kDa), rabbit muscle phosphorylase b (97.4 kDa), bovine serum albumin (66 kDa), egg albumin (45 kDa), carbonic anhydrase (31 kDa), trypsin inhibitor (21.5 kDa), lysozyme (14.4 kDa), and aprotinin (6.5 kDa).

ACE assay

ACE inhibitory activity was measured by the method of Cushman and Cheung (13) with modifications established in our laboratory (14,15). The reaction mixture contained 150 L of 5 mM Hip-His-Leu as a substrate, 50 L of rabbit lung ACE powder (5 munit) in a 50 mM sodium borate buffer (pH 8.3), and 50 L of the sample solution. The reaction was carried out at 37°C for 30 min, and terminated by adding 250 L of 1 N HCl, and 1 mL of ethylacetate. After centrifugation, the absorbance of the supernatants was measured at 228 nm. The IC_{50} value was defined as the concentration of ACE inhibitor required to inhibit 50% of ACE activity.

Peptide sequencing

ACE inhibitory peptide sequence was determined by automated Edman degradation using a 476A Protein sequencer (Applied Biosystem, Inc., Forster City, CA, USA).

RESULTS AND DISCUSSION

Preparation of duck meat protein hydrolysates

Duck meat protein hydrolysates were prepared by enzymatic hydrolysis using trypsin at 37°C for 6 hrs after separating the muscle into wing, breast, and thigh parts. A preliminary experiment, using various proteases, found that trypsin was the most suitable protease for duck meat hydrolysis (data not shown). Available amino group concentrations of enzymatic hydrolysates of duck meat protein, which is an indicator of degree of hydrolysis, was determined using the TNBS assay (Fig. 1). Fig. 1 shows that 2 hrs of hydrolysis was sufficient for preparation of hydrolysates and that thigh meat was the most suitable for further study based on the degree of hydrolysis; although the ACE assay indicated that ACE inhibitory activity was almost the same for all the meat parts (data not shown). Therefore, thigh meat was selected for further study. Fig. 2 shows the SDS-PAGE profile of duck meat protein hydrolysates. After 2 h of hydrolysis the major protein bands of 35 kDa, 27 kDa, and 12 kDa were degraded into smaller bands below 10 kDa. The degree of hydrolysis is usually dependent upon various factors, including: the type of protease, hydrolysis conditions such as pH and temperature, and hydrolysis time. We have previously reported the optimal experimental conditions for hydrolysis of several food proteins (16-18). Under the experimental conditions used in this study, 2 hr of trypsin hydrolysis at 37°C was sufficient for the preparation of duck meat protein hydrolysates.

Purification of an ACE inhibitory peptide

Duck meat protein hydrolysates were filtered using YM-

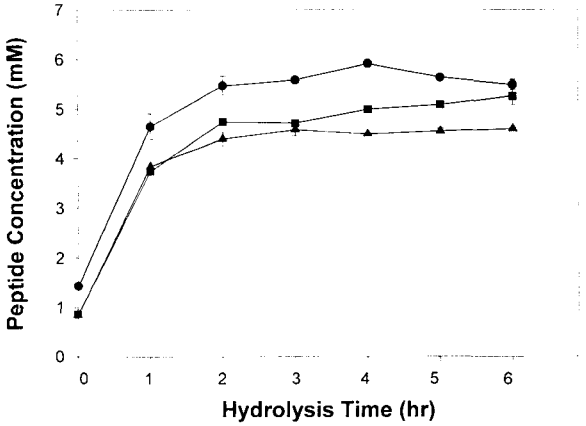

Fig. 1. Effect of hydrolysis time on the peptide concentrations of trypsin hydrolysates.
●: Thigh, ■: Wing, ▲: Breast.

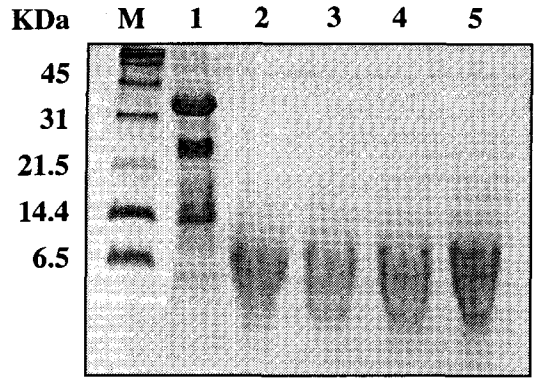

Fig. 2. SDS-PAGE profile of duck meat protein hydrolysates. M: protein molecular weight marker.
Lane1: hydrolysis time 0 hr, 2: 1 hr, 3: 2 hrs, 4: 3 hrs, 5: 6 hrs.

1 (Mw 1,000 cut-off) and YM-3 membranes (Mw 3,000 cut-off). ACE inhibition of the YM-3 filtrate was higher than that of the YM-1 filtrate (data not shown). Therefore, the YM-3 filtrates, rich in ACE inhibitory peptides, were fractionated using anion exchange chromatography. There were three major peaks on the QAE Sephadex chromatogram (Fig. 3). Among them, the F3 fraction had the highest inhibitory activity (65%), and was pooled and further purified using Sephadex G-15 gel permeation chromatography (Fig. 4), revealing four major peaks. Among the four peaks, the F32 fraction had the highest ACE inhibitory

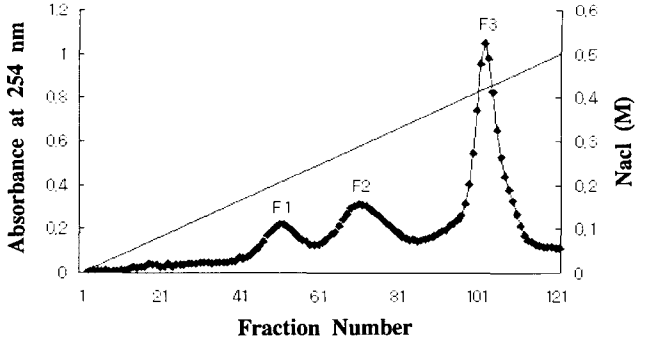

Fig. 3. QAE sephadex chromatogram of duck meat protein hydrolysates.

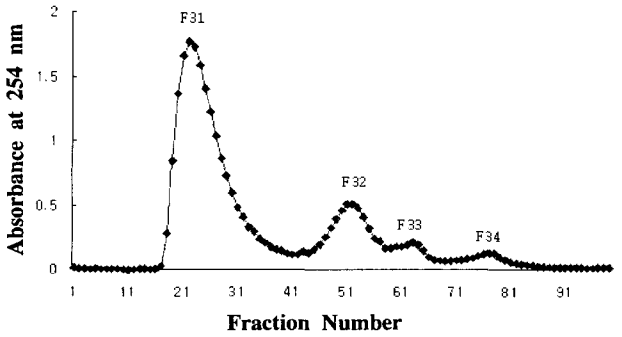

Fig. 4. Sephadex G-15 gel permeation chromatogram using F3 of Fig. 3.

activity (87%) and was selected and loaded onto FPLC with a Resource Q column for further purification. We were able to obtain one major peak (F321) from the gradient elution (Fig. 5) from which a novel ACE inhibitory peptide was purified by normal phase HPLC using an NH_2 column (Fig. 6). During the gradient elution, a minor peak exhibiting ACE inhibition, F3211, was also isolated. It came out at 40% of solvent B. ACE inhibition of the peak had a 85.9 M as IC_{50} value. The ACE inhibitory peptide from the F3211 fraction was analyzed using a protein sequencer and identified as a tetrapeptide, Glu-Asp-Leu-Glu. The tetra-peptide we isolated and sequenced has never been reported as an ACE inhibitory peptide. Nakashima et al. (19) reported the identification of ACE inhibitory peptides from porcine skeletal muscle protein hydrolysates. The major peptides they purified were Met-Asn-Pro,

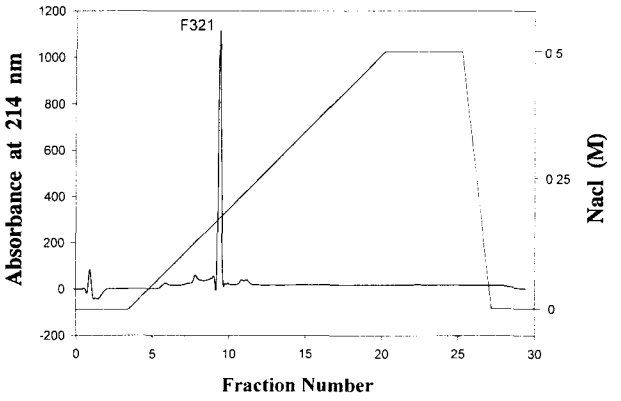

Fig. 5. Anion exchange chromatogram using F32 in Fig. 4.

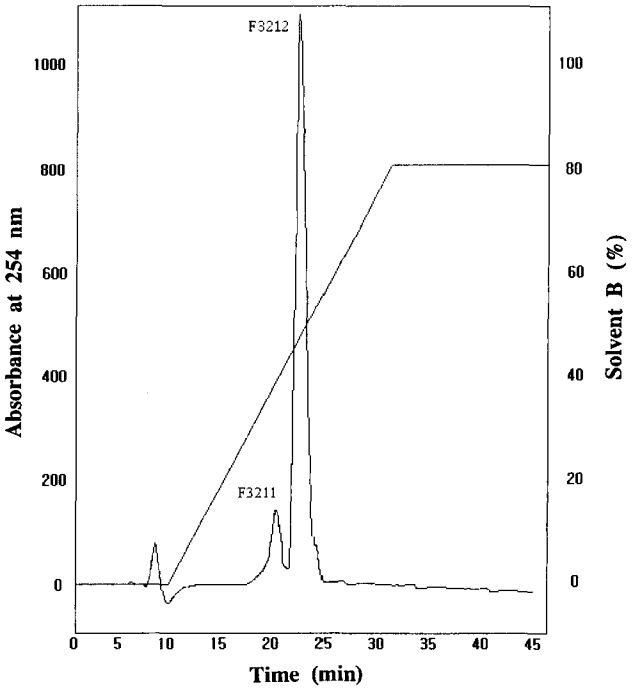

Fig. 6. Normal phase HPLC chromatogram using F321 in Fig. 5.

Thr-Asn-Pro, Asn-Pro-Pro, and Ile-Thr-Thr-Asn-Pro. ACE inhibitory activity of the peptide purified in this study is relatively low compared with some other peptides like Leu-Arg-Pro or Ile-Trp (6,7). However, the ACE inhibitory peptides isolated from porcine skeletal muscle protein hydrolysates, which have similar or lower inhibitory activities than the tetrapeptide in this study, exhibited potent antihypertensive activities (19). Recently, foods such as sour milk and soup containing ACE inhibitory peptides have been marketed in Japan (19). Therefore, the peptide purified in this study is a possible candidate for application as a dietary supplement or as a component of a functional food product. Additional work is needed to elucidate the structure-activity relationship of the ACE inhibitory peptide and future research should include *in vivo* experiments to characterize the physiological functions of this food-derived bioactive peptide.

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