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Purification and identification of angiotensin I-converting enzyme inhibitory peptide from buckwheat (*Fagopyrum esculentum* Moench)

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Abstract

Angiotensin I-converting enzyme (ACE) inhibitory peptide was isolated and identified from buckwheat (*Fagopyrum esculentum* Moench). Buckwheat protein extract was prepared by stirring in water (pH 9.0) for 30 min, followed by centrifugation at 15,000*g* for 20 min. The protein extract was then filtered using an YM-10 membrane. An ACE inhibitor was purified using consecutive chromatographic methods including: ion-exchange chromatography, gel filtration chromatography, and reverse-phase high performance liquid chromatography. The ACE inhibitor was identified to be a tripeptide, Gly-Pro-Pro, having IC_{50} value of 6.25 μg protein/ml, by protein sequencing system and electrospray-LC–mass spectrometry.

Keywords: Buckwheat; *Fagopyrum esculentum* Moench; Angiotensin I-converting enzyme inhibitor; Bioactive peptide

1. Introduction

In recent years, food intake is increasingly being considered not only as a source of nutrients but also as a source of bioactive compounds, including bioactive peptides. These peptides may already be present in foods as natural components or may be produced after hydrolysis by chemical or enzymatic treatments (Vioque et al., 2000). Biological peptides with antihypertensive activity by inhibition of the angiotensin I-converting enzyme (ACE; peptidyldipeptide hydrolase; EC 3.4.15.2) have been described. ACE is a Zn–metallopeptidase and plays an important role in regulating blood pressure. ACE catalyzes the conversion of an inactive form, decapeptide (angiotensin I) to a potent vasoconstrictor octapeptide (angiotensin II) and also inactivates the antihypertensive vasodilator bradykinin (Ondetti, Rubin, & Cushman, 1997). The naturally occurring peptides with ACE inhibitory activity were first obtained from snake venom (Ondetti et al., 1971). These ACE inhibitors contained 5–13 amino acid residues per molecule, and most of them had a C-terminal sequence of Ala-Pro or Pro-Pro. Oshima, Shimabukuro, and Nagasawa (1979) reported ACE inhibitory peptides produced from food proteins by digestive protease. Afterward, many other ACE inhibitory peptides have been discovered from food or from enzymatic digestion of food proteins. These food protein sources include fish (Byun & Kim, 2001; Curis, Dennes, Waddell, Macgillivray, & Ewart, 2002; Fahmi et al., 2004), chickpea (Pedroche et al., 2002; Yust et al., 2003), and mushroom (Choi, Cho, Yang, Ra, & Suh, 2001; Lee, Kim, Park, Choi, & Lee, 2004). Recently, many researchers have studied inhibitory activities on ACE and antihypertensive effects after oral or intravenous administration in animal experiments and in clinical trials (Sato et al., 2002; Seppo, Jauhiainen, Poussa, & Korpela, 2003; Shin et al., 2001).

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Buckwheat (*Fagopyrum esculentum* Moench) is an herbaceous plant of the Polygonaceae family. In some countries such as China, Japan, and Poland, buckwheat is recognized as a valuable source of so-called "functional food" (Dietrych-Szostak & Oleszek, 1999). Buckwheat is a very interesting species, because of the high nutritive value of its seed proteins. Therefore, buckwheat is receiving significant attention owing to several potential health benefits arising from its strong hypocholesterolaemic activity (Kayashita, Shimaoka, Nakajoh, Yamajaki, & Nato, 1997), and cholesterol- and blood pressure-reducing effect (He et al., 1995). In Korea, buckwheat has been grown for centuries and used both as a food and a traditional medicine.

Our studies (Kim, Lee, Do, Shin, & Yang, 2000; Kwon, Lee, Shin, & Yang, 2000; Lee, Kwon, Shin, & Yang, 1999; Park et al., 2000) revealed the efficacy of natural ACE inhibitors and protein hydrolysates in reducing blood pressure. Almost all the natural hypotensive ACE inhibitors are peptides, because of their high competitive affinity with the ACE active site. Therefore, the objectives of this study were to isolate ACE inhibitory substance from buckwheat (*F. esculentum* Moench) and to identify a new ACE inhibitory peptide.

2. Materials and methods

2.1. Materials

Buckwheat (*F. esculentum* Moench) was purchased from Kyung-Dong market in Seoul, Korea. The buckwheat was ground and extracted with diethyl ether in a shaker for 24–48 h in order to remove most of the fat content. The defatted buckwheat flour was used as the starting material for isolation of ACE inhibitor. The ACE enzyme powder from rabbit lung acetone extract and hippuryl-L-histidyl-L-leucine (HHL) were purchased from Sigma Chemical Co. (St. Louis, MO, USA). All other chemicals were of HPLC or analytical grade.

2.2. Assay of ACE inhibitory activity

ACE inhibitory activity was measured by the method of Cushman and Cheung (1971) with modification. The reaction mixture contained 50 μl of 20 mM HHL as a substrate, 50 μl of ACE powder (20 munit) in a 150 mM sodium borate buffer (pH 8.3), and 50 μl of the sample solution. The reaction was carried at 37 °C for 30 min, and terminated by adding 100 μl of 1.75 N HCl and 1 ml of ethyl acetate. After centrifugation, the absorbance of the supernatants was measured at 228 nm. The IC_{50} value, defined as the concentration of the peptide inhibits 50% of the ACE activity, was determined by measuring the ACE inhibitory activity and peptide contents of each sample after the regression analysis. The peptide contents were measured using the method of Bradford (Bradford, 1976).

2.3. Preparation of buckwheat protein extract

The defatted buckwheat flour (1 kg) was stirred in 10 l of water (adjusting the pH 9.0 with 2 N NaOH)

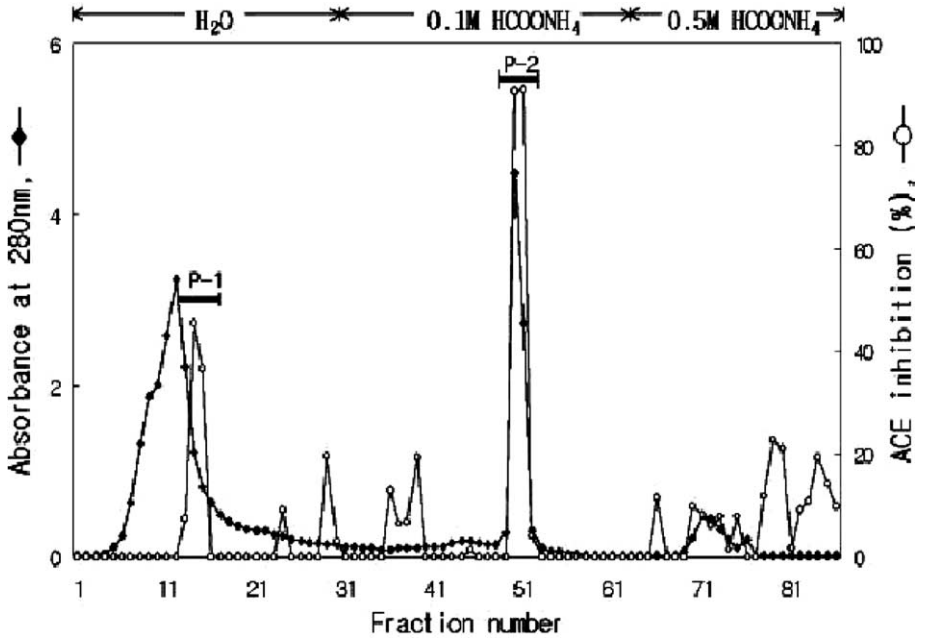

Fig. 1. Sephadex C-25 column chromatography profile and ACE inhibitory activity using buckwheat protein filtrate from an YM-10 membrane. Elution was done in a stepwise gradient of 500 ml of water, 400 ml of 0.1 M ammonium formate, and 400 ml of 0.5 M ammonium formate at a flow rate of 8.0 ml/min.

for 30 min and the extract was centrifuged at 15,000*g* for 20 min. The protein in the supernatant was precipitated by adjusting the pH to 4.5 (around pI of buckwheat protein) with 2 N HCl, and the protein precipitate was separated by centrifugation at 15,000*g* for 20 min. The supernatant was freeze-dried and used for further study.

2.4. Isolation of ACE inhibitor

Buckwheat protein was divided into large and small (less than 10,000 Da) molecular weight fractions through ultrafiltration at 4 °C using an YM-10 membrane (MWCO, 10000; Amicon Co., Beverly, MA, USA) (Maruyama, Miyoshi, & Tanaka, 1989). The fractions were

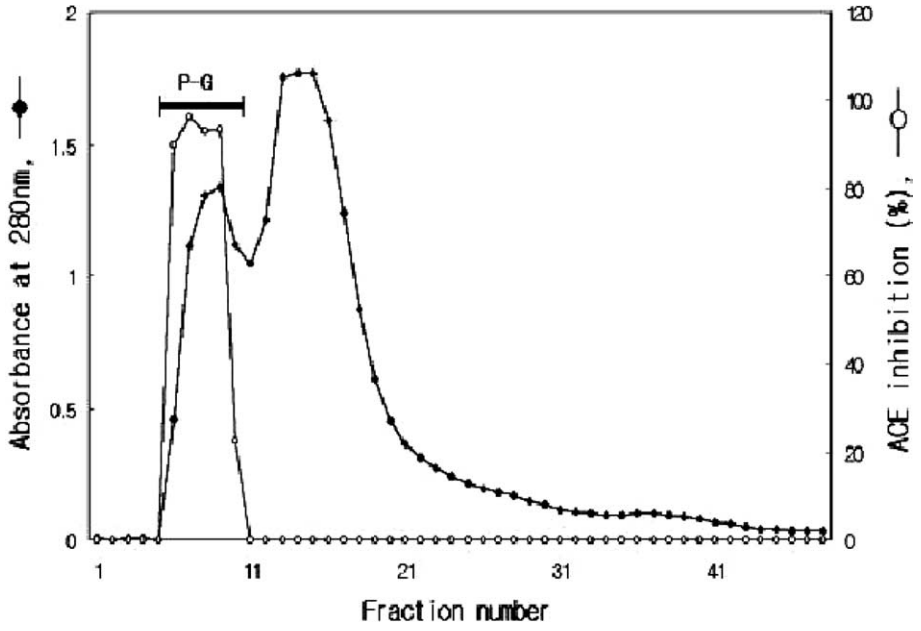

Fig. 2. Sephadex G-10 column chromatography profile and ACE inhibitory activity using active fraction P-2 from Sephadex C-25 column chromatography. Elution was done with water at a flow rate of 5.0 ml/min.

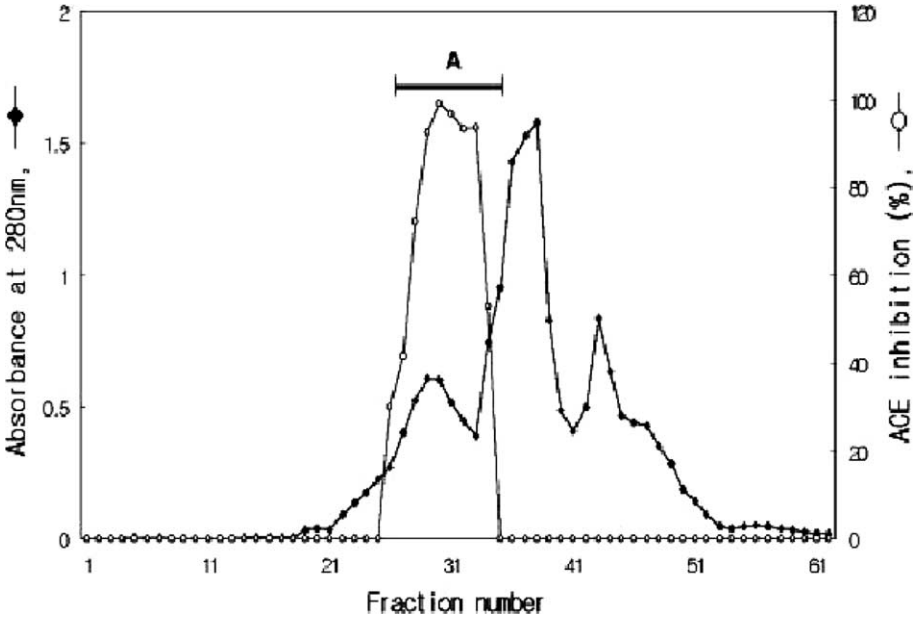

Fig. 3. Sephadex LH-20 column chromatography profile and ACE inhibitory activity using active fraction P-G from Sephadex G-10 column chromatograpy. Elution was done with 10% methanol at a flow rate of 8.0 ml/h.

subjected to consecutive chromatographic separations using Sephadex C-25, Sephadex G-10, Sephadex LH-20, and reverse phase HPLC.

The peptide solution was then loaded in a Sephadex C-25 ion exchange column (3.3 × 16 cm; Pharmacia Co., Uppsala, Sweden) pre-equilibrated with water and eluted at a flow rate of 8 ml/min. Samples were eluted in a series as follows: 500 ml of water, 400 ml of 0.1 M ammonium formate, and 400 ml of 0.5 M ammonium formate. The eluate was monitored by the absorbance at 280 and 228 nm, respectively (Maruyama, Nakagomi, Tomizuka, & Suzuki, 1985). The highest inhibitory fraction was further isolated using a Sephadex G-10 column (1.6 × 20 cm; Pharmacia Co., Uppsala, Sweden) and eluted at a flow rate of 5 ml/min with water (Kohama et al., 1988). The highest inhibitory fraction was further purified using a Sephadex LH-20 column (2.0 × 80 cm; Pharmacia Co., Uppsala, Sweden) pre-equilibrated with 10% methanol and eluted at a flow rate of 8 ml/h with the same solvent (Kohama et al., 1989). Finally, to further purify an ACE inhibitor, the highest inhibitory fraction in gel filtration chromatography was applied to a HPLC reverse-phase column system (Capcell pak C_{18} column, 10 × 250 mm; Shiseido, Tokyo, Japan). Elution was carried out using a linear gradient of acetonitrile (5–20%) and water (95–80%) at a flow rate of 3.0 ml/min. The eluate was monitored at 210 nm and fractions or individual peaks were collected for assay of ACE inhibitory activity.

2.5. Identification of ACE inhibitory peptide

The molecular mass of the purified ACE inhibitor was determined using liquid chromatography–mass spectrometry (LC–MS) (Platform II, Micromass, Manchester, UK) with electrospray mode. The data were collected with the MCA (multi-channel acquisition) method with a single injection. The solvent was 0.2% formic acid solution (water:acetone = 1:1) (Kim, Lee, & Kwon, 1999). ACE inhibitory peptide sequence was

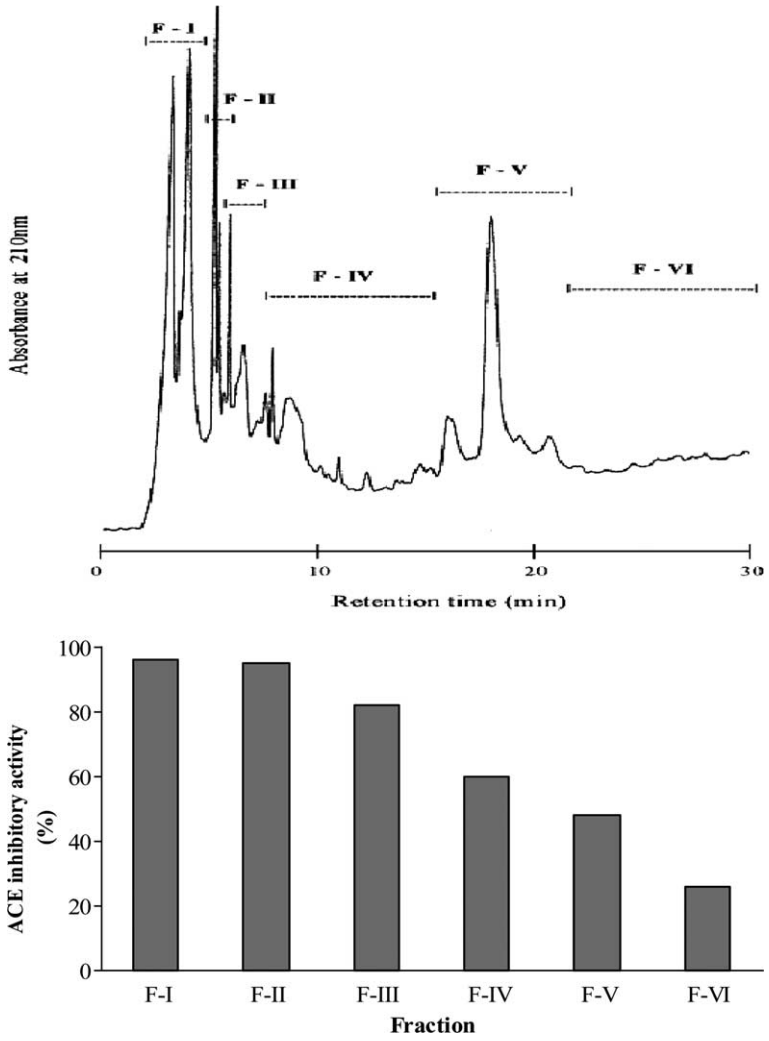

Fig. 4. HPLC profile and ACE inhibitory activity using active fraction A from Sephadex LH-20 column chromatography. Elution was done in a linear gradient condition of acetonitrile (5–20%) and water (95–80%) at a flow rate of 3.0 ml/min.

identified through the Edman degradation (Allen, 1989) using the liquid phase peptide sequenator (Procise™, Perkin–Elmer, Protein sequencing system, Foster, CA, USA).

3. Results and discussion

3.1. Isolation of ACE inhibitor

The protein extract of buckwheat (*F. esculentum* Moench) was obtained by steeping in water (pH 9.0) and then its inhibitory activity was measured . The inhibitory activity of the buckwheat protein against ACE was 93.3% and its IC_{50} value was 70.5 μg protein/ml.

To isolate the ACE inhibitory peptide, the buckwheat protein extract was filtered through an YM-10 membrane. The low molecular weight fraction (less than 10,000 Da) in the ultrafiltration of buckwheat protein had higher ACE inhibitory activity than the high molecular weight fraction. Noh and Song (2001) previously reported that membrane filtration with a 1000 Da molecular weight cut-off could be a useful processing method for the purification of ACE inhibitors, because most potent ACE inhibitory substances have molecular weights below or around 1000 Da (Ariyosh, 1993). The filtrate from an YM-10 membrane of buckwheat protein was concentrated and loaded in Sephadex C-25 ion-exchange column, and resolved into two major fractions (Fig. 1). Among them, the P-2 fraction exerted the highest inhibitory activity of 91.0% (IC_{50} value of 25.7 μg protein/ml). Therefore, the P-2 fraction was then collected, concentrated and used for further isolation by Sephadex G-10 column chromatography. The eluate was divided into two fractions from the column. As observed in Fig. 2, the highest inhibitory activity (96.0%, IC_{50} value of 21.3 μg protein/ml) was found in the fractions including the peptides with higher molecular masses. The P-G fraction was pooled and subjected to Sephadex LH-20 column chromatography. Among three peaks, the higher molecular weight fraction eluted at earlier time had 95.0% of the ACE inhibitory activity (IC_{50} value of 15.1 μg protein/ml). On the other hand, the lower molecular weight fractions had rare ACE inhibitory activity (Fig. 3). The highest inhibitory fraction was used for the following series of HPLC separation for purification of the ACE inhibitory peptide. For removing the impurities of the selected peptides, the peptide solutions with inhibitory activity were first filtered through 0.4-μm membranes. The peptide solution was then applied to a HPLC Capcell pak C_{18} reverse-phase column, and the peptides were collected in six fractions that were assayed for ACE inhibitory activity (Fig. 4). Among six fractions, fractions F-I and F-II possessed high inhibitory activities of 96% and 95%, respectively. To obtain a single peptide, the fractions F-I and F-II were pooled and subjected to further purification work using HPLC. The peptide was found to be relatively pure, although the chromatogram still showed a small shoulder behind the peak (Fig. 5). Since the separation of peptides is known to be very difficult due to the complexities of peptides, electrophoresis, TLC, and HPLC or their combination were attempted for a better separation (Pedroche et al., 2002).

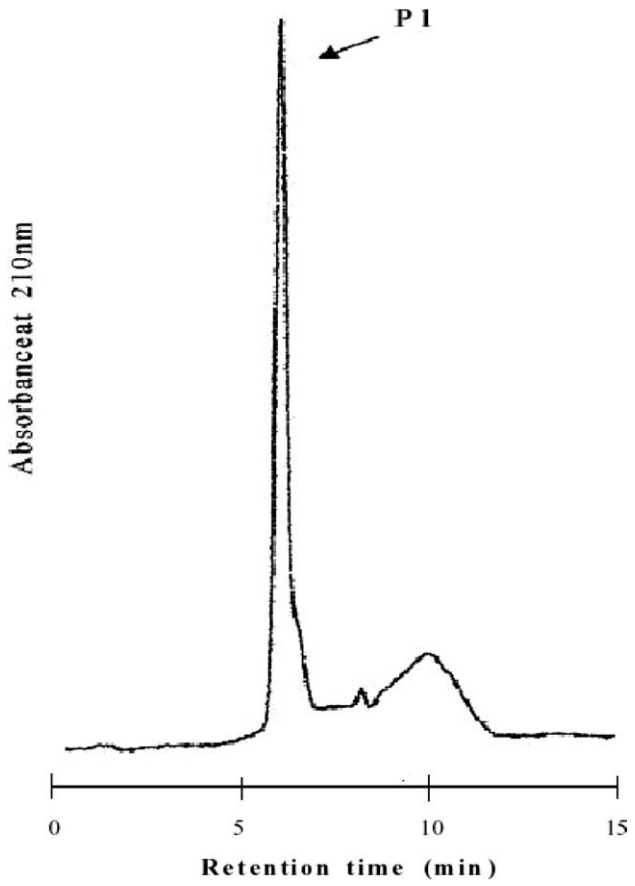

Fig. 5. HPLC profile of the purified ACE inhibitory peptide from *Fagopyrum esculentum* Moench. Elution was done in linear gradient conditions of acetonitrile (5–20%) and water (95–80%) at a flow rate of 3.0 ml/min. Arrow P-1 indicates a peptide peak exhibiting the highest ACE inhibitory activity.

Table 1
Purification and ACE inhibitory activity (IC_{50}) of purified peptides from *Fagopyrum esculentum* Moench

Purification step	IC_{50} (μg protein/ml)[a]	Purification fold[b]
Protein extract	70.5	1.0
Ultrafiltration	32.3	2.2
Sephadex C-25	25.7	2.7
Sephadex G-10	21.3	3.3
Sephadex LH-20	15.1	4.7
RP-HPLC	6.3	11.3

[a] IC_{50} defined as the concentration which inhibits 50% of the angiotensin-I converting enzyme activity.
[b] Relative value of reciprocal of ACE IC_{50}.

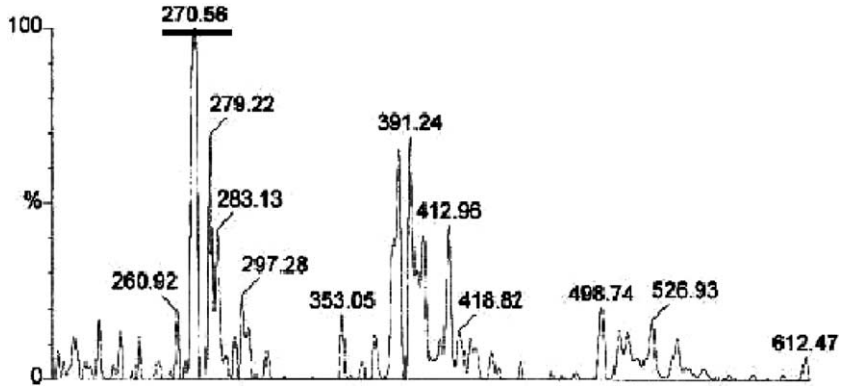

Fig. 6. Mass spectrum of the purified ACE inhibitor from *Fagopyrum esculentum* Moench.

Table 1 shows the summary for the purification of ACE inhibitor from buckwheat (*F. esculentum* Moench).

3.2. Identification of ACE inhibitory peptide

The inhibitory substance was identified by mass spectrometry and peptide sequenator. Its molecular weight was determined to be 270.56 by ESI mass spectrum (Fig. 6). The ACE inhibitory peptide was then analyzed using a peptide sequenator and was identified to be a tripeptide, Gly-Pro-Pro, having IC_{50} value of 6.25 μg protein/ml. Cheung, Wang, Ondetti, Sabo, and Cushman (1980) concluded that the C-terminal amino acid of peptides made the most important contribution to substrate binding at the ACE active site and that the most favorable C-terminal amino acids were tryptophan, tyrosine, phenylalanine, and proline. The present results indicated that the ACE inhibitory peptide was proline, which is in agreement with Cheung et al. The strong ACE inhibitory activity of Gly-Pro-Pro isolated in this study supported the importance of proline at the carboxyl terminal. Li, Matsui, Matsumoto, Yamasaki, and Kawasaki (2002) also reported the ACE inhibitory peptides isolated from buckwheat protein. They isolated 4 tripeptides: Tyr-Gln-Tyr, Pro-Ser-Tyr, Leu-Gly-Ile, and Ile-Thr-Phe. Among them, Tyr-Gln-Tyr had the lowest IC_{50} value of 4 μM, which was stronger than the peptide (23.1 μM) purified in this work. However, the amino acid sequence of their peptides and their parents were unique making comparisons difficult. Moreover, inhibitory potency may be very variable between peptides or protein sources (Ariyosh, 1993). Nevertheless, this IC_{50} value is still far from the IC_{50} value of the synthetic ACE inhibitor captopril (0.041 μM) (Megias et al., 2004).

In conclusion, the ACE inhibitory peptides with high activities could be derived from protein of *F. esculentum* Moench, which have been traditionally used as a Korean medicine treatment for patients having hypertension. This result demonstrates that the inhibitory peptide may be useful as a functional food ingredient with anti-hypertensive property. Therefore, further studies on animal experiments or clinical trials need to be performed.

Acknowledgment

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