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Tyr-Pro-Lys, an angiotensin I-converting enzyme inhibitory peptide derived from broccoli (*Brassica oleracea Italica*)

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Abstract

To investigate the naturally occurring angiotensin I-converting enzyme (ACE) inhibitor, broccoli (*Brassica oleracea Italica*) extracts were used for its isolation and identification. After treatment with 50% acetone for membrane breakdown, ethyl acetate, *n*-butanol, and water were used for the preparation of broccoli extracts. The water-soluble extract from broccoli had 76.9% ACE inhibitory activity, while those of other organic solvent extracts showed lower ACE inhibitory activities. An ACE inhibitory peptide was isolated using column chromatographic methods including: Amberlite XAD-4, Sephadex LH-20, and high performance liquid chromatography. The purified ACE inhibitory peptide was identified to be a tripeptide, Tyr-Pro-Lys, having an IC_{50} value of 10.5 μg protein/ml.

Keywords: Broccoli; *Brassica oleracea Italica*; Angiotensin I-converting enzyme inhibitor; Bioactive peptide

1. Introduction

Angiotensin I-converting enzyme (ACE, kinase II, EC 3.4.15.1) is potentially of great importance for controlling blood pressure by virtue of the rennin-angiotensin system. This enzyme cleaves the C-terminal of histidyl-leucine of the inactive decapeptide angiotensin I to form the octapeptide angiotensin II, a potent vasoconstrictor (Hollenberg, 1979; Ondetti & Cushman, 1982; Gohlke et al., 1994). Since the original discovery of ACE inhibitors in snake venom (Ondetti et al., 1971), captopril (d-3-mercapto-2-methylpranory-1-proline), enalapril, and lisinopril, effective oral inhibitors, have been developed and all are currently used as clinical antihypertensive drugs (Ondetti, Rubin, & Cushman, 1997). Although synthetic ACE inhibitors, including captopril, are remarkably effective as antihypertensive drugs, they cause adverse side effects. Therefore, research and development to find safer, economical, and naturally occurring ACE inhibitors are desirable for the prevention and remedy of hypertension. Food-derived ACE inhibitory peptides have been isolated from food or from enzymatic digestion of food proteins, including buckwheat (Li, Matsui, Matsumoto, Yamasaki, & Kawasaki, 2002), chickpea (Pedroche et al., 2002; Yust et al., 2003), garlic (Suetsuna, 1998), mushroom (Choi, Cho, Yang, Ra, & Suh, 2001; Lee, Kim, Park, Choi, & Lee, 2004), spinach (Yang, Marczak, Yokoo, Usui, & Yoshikawa, 2003), and sunflower (Megias et al., 2004). Recently, many researchers have studied in vivo inhibitory activities on ACE and antihypertensive effects after oral or intravenous administration in animal experiments and in clinical trials (Fujita, Yamagami, & Ohshima, 2001; Shin et al., 2001; Wu & Ding, 2001; Sato et al., 2002; Seppo, Jauhiainen, Poussa, & Korpela, 2003).

In the last few years, broccoli (*Brassica oleracea Italica*) cultivation and consumption have increased

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markedly worldwide. This increase could be attributed to its potential health benefits because of the high nutritive value of vitamin C and glucosinolates, having anti-carcinogenic properties (Dillard & German, 2000). Also, broccoli is well known to be a protein-rich vegetable, however few scientific studies have been performed on its protein. Previously, we have isolated and identified the ACE inhibitory substances from various foods (Lee, Kwon, Shin, & Yang, 1999; Kim, Lee, Do, Shin, & Yang, 2000; Kwon, Lee, Shin, & Yang, 2000; Park et al., 2000). Almost all the natural hypotensive ACE inhibitors are peptides, because of their high competitive affinity with the ACE active site. Therefore, our objectives were to (1) examine for ACE inhibitory activity, (2) isolate ACE inhibitory substance, and (3) identify a new ACE inhibitor from broccoli (*Brassica oleracea Italica*).

2. Materials and methods

2.1. Materials

Commercially available broccoli (*Brassica oleracea Italica*) used for the experiments was produced in Chonan (Choongnam, Korea). After hot-air drying and then grinding the broccoli with a ball miller (WHF-714, Wonjin Co., Seoul, Korea), broccoli powder was sealed and stored at 4 °C. The ACE enzyme powder from rabbit lung acetone extract and substrate for ACE, hippuryl-L-histidyl-L-leucine (HHL), were obtained 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 some 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 that 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 (1976).

2.3. Preparation of water and solvent extracts

For membrane breakdown, 50% acetone solution (20 l) was added into 2 kg of broccoli powder and then sonicated for 40 min. The mixture was filtered and concentrated using a rotary evaporator (Rotavapor, Buchi, Flawel, Switzland). The separation procedure through solvent extraction was also done in advance (Kijima, Ide, & Otsuka, 1995). Water-soluble and organic (ethyl acetate and *n*-butanol) solvent-soluble extracts were obtained from 50% acetone extract.

2.4. Isolation of ACE inhibitor

The ACE inhibitor from broccoli was purified using a series of the following column chromatography procedures by a modification of the methods of Cho, An, and Choi (1993), Ahn, Bae, and Choi (1996), and Park, Ha, and Park (1996). The peptide solution was then loaded in an Amberlite XAD-4 column (4.5 × 32 cm, Pharmacia Co., Uppsala, Sweden) equilibrated with water. Samples were eluted in a series as follows: 400 ml of water, 300 ml of 30%, 50%, and 70% methanol, and 800 ml of 100% methanol. The collected fraction was concentrated with a rotary evaporator and the ACE inhibitory activity was measured. The highest inhibitory fraction was further purified using a Sephadex LH-20 column (3.5 × 21 cm, Pharmacia Co., Uppsala, Sweden) equilibrated with water and eluted in a series (150 ml of water, 100 ml of 5%, 10%, 15%, 20%, 30%, and 50% methanol) at a flow rate of 8 ml/h. 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.

Confirmation of the products in the assay reaction was carried out through thin layer chromatography of the reaction mixture. The plates (HPTLC silicagel 60F_{254}, Merck Co., Germany) were developed in chloroform/methanol/water (40/16/3) mixture. After developing for 40 min, the developed plate was sprayed with ninhydrin solvent and then dried (110 °C, 15 min) in a vacuum drying oven.

2.5. Identification of ACE inhibitory peptide

The amino acid composition of ACE inhibitor from broccoli was analyzed using a PICO-TAG™ HPLC (Hewlett Packard HPLC 1050, Palo Alto, USA) after hydrolysis in 6 N HCl at 110 °C for 24 h and derivatization with phenylisothiocyanate (Chang, Skarge, & Satterlee, 1989). The sequence of the purified ACE inhibitory peptide from broccoli was identified through the Edman degradation (Allen, 1989) method using a liquid phase peptide sequenator (Procise™, Perkin–Elmer, Applied Biosystem, Foster, CA, USA).

3. Results and discussion

3.1. ACE inhibitory activity of water and solvent extracts

To elucidate the characteristics of the active peptide, we separated the extracts into three solvent systems: ethyl acetate, *n*-butanol, and water according to their polarities. The ACE inhibitory activity of 50% acetone extract was 63.3% ($IC_{50} = 55.09$ μg protein/ml). Among the extracts from 50% acetone extract, the water-soluble extract had high ACE inhibitory activity (76.9%, $IC_{50} = 29.25$ μg protein/ml) and yield (69.5%), while those of other organic solvent extracts were lower than the water-soluble extract. The results revealed that the water extraction was the best method for ACE inhibitory substances, suggesting that ACE inhibitory substance may be a water-soluble substance such as protein. Therefore, the water-soluble extract was selected for further study.

3.2. Isolation of ACE inhibitor substance by column chromatography from the column

Using water-soluble extracts, an Amberlite XAD-4 column was run with water and 30%, 50%, 70%, and 100% methanol for elution and each fraction was measured for ACE inhibitory activity (Fig. 1). The ACE inhibitory activities of 50%, 70%, and 100% methanol fractions were 84.4%, 82.8%, and 81.8%, respectively. These methanol fractions had relatively higher yield than others. Thus, each collected fraction of 50%, 70% and 100% methanol in an Amberlite XAD-4 column was used for the subsequent chromatography.

Using these methanol fractions, Sephadex LH-20 column chromatography was carried out for further separation. The eluate was divided into four (F1, F2, F3, F4) major fractions from the column (Fig. 2). Among them, the F1 (82.5%) and F2 (57.9%) fractions had high ACE inhibitory activities with the F1 fraction having the highest yield. Thus, the F1 and F2 fractions were mixed, evaporated, and used for the re-chromatography. After re-chromatography, three fractions (F1-1, F1-2, F1-3) which showed ACE inhibitory activities were collected

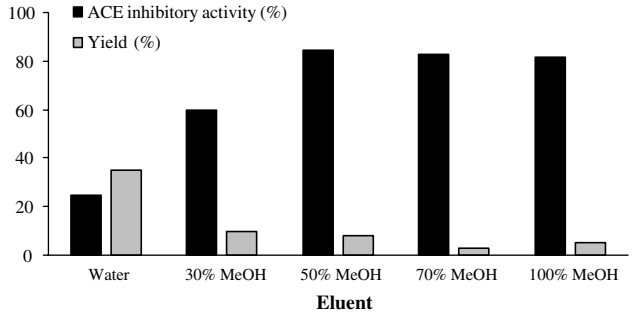

Fig. 1. ACE inhibitory activity and yield of the eluate obtained by an Amberlite XAD-4 column using broccoli water extract.

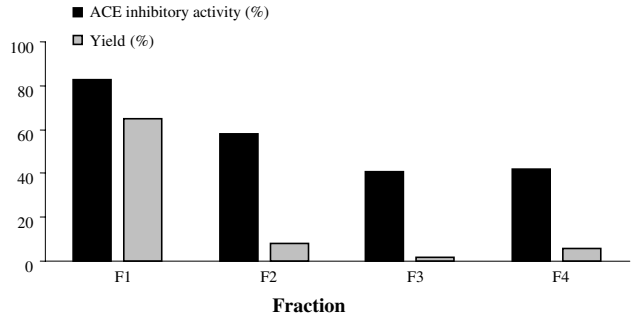

Fig. 2. ACE inhibitory activity and yield of the fraction obtained by a Sephadex LH-20 column using the 50–100% methanol eluate in Fig. 1.

(Fig. 3). The F1-1 and F1-2 fractions had strong ACE inhibitory activities, however, the F1-2 fraction showed low yield. The F1-1 fraction, having the highest activity (86.5%, $IC_{50} = 20.14$ μg protein/ml) and yield was subjected to further analysis.

To obtain a single compound, further isolation and purification work were performed through HPLC after removing the impurities of the selected peptides (F1-1 fraction) using a HPLC pre-filter of 0.4 μm pore size. As shown in Figs. 4 and 5(a), six fractions (P-1, P-2, P-3, P-4, P-5, P-6) were collected according to the retention time and the ACE inhibitory activities were measured. The P-2 fraction had the highest activity (92.4%, $IC_{50} = 18.39$ μg protein/ml) and yield (45.5%). After re-chromatography of the active P-2 fraction,

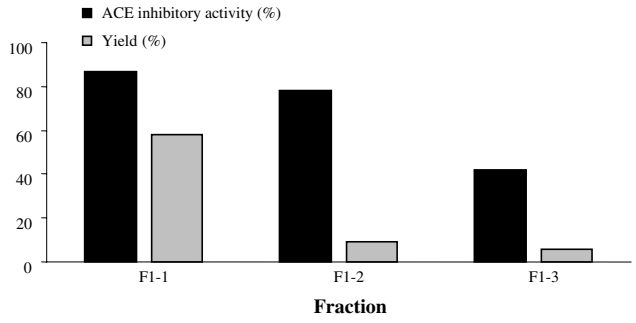

Fig. 3. ACE inhibitory activity and yield of the fraction obtained by a Sephadex LH-20 column using the F1 and F2 in Fig. 2.

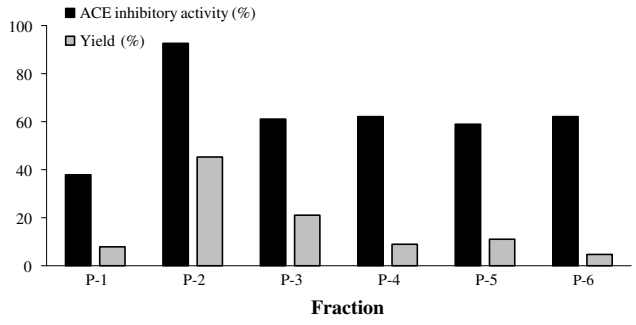

Fig. 4. ACE inhibitory activity and yield of the fraction obtained by a Capcell pak C18 column using the F1-1 in Fig. 3.

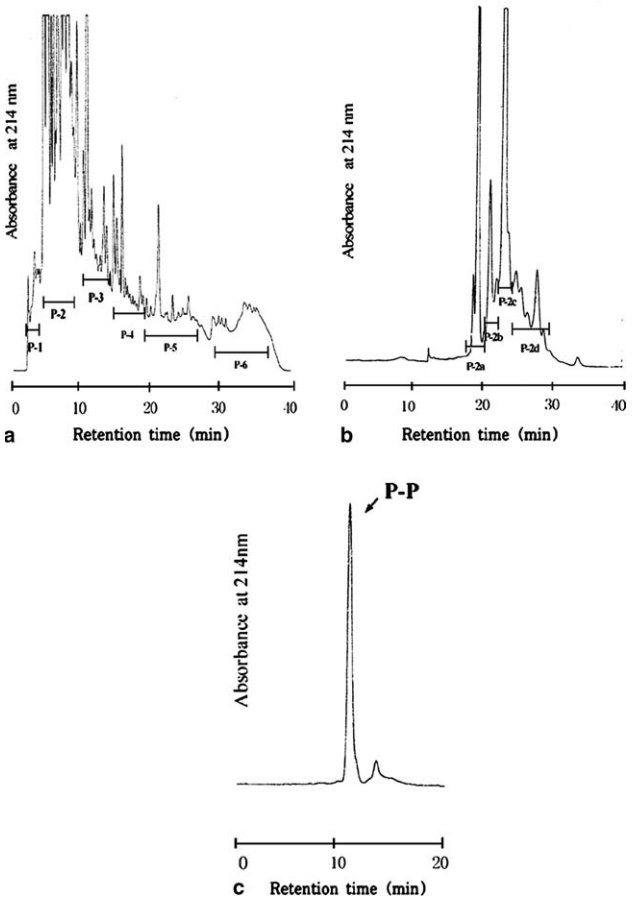

Fig. 5. Purification of ACE inhibitory peptide from fraction F1-1 (a), fraction P-2 (b), and fraction P-2c (c) by HPLC. Arrow P-P indicates the purified ACE inhibitory peptide.

the P-2c fraction was pooled (Fig. 5(b)) and applied to a HPLC Capcell pak C_{18} reverse-phase column for isolation of a single pure compound P-P (Fig. 5(c)). The peptide was found to be relatively pure, although the chromatogram still showed a small shoulder behind the peak. 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).

To confirm the active inhibitory substances in the HPLC fractions, TLC was run and then developed with ninhydrin reagents. All fractions and the single pure compound showed a positive reaction (data not shown), indicating this ACE inhibitory substance to be a peptide compound.

Table 1 shows the summary for the purification of ACE inhibitor from broccoli (*Brassica oleracea Italica*).

3.3. Identification of ACE inhibitory peptide

As observed in Fig. 6, the amino acid sequence of the active peak (P-P), which had a high ACE inhibitory

Table 1
Purification and ACE inhibitory activity (IC_{50}) of purified peptides from *Brassica oleracea Italica*

Purification step	IC_{50}[a] (μg protein/ml)	Purification fold[b]
50% Acetone extract	55.09	1.00
Water extract	39.25	1.40
Amberlite XAD-4	25.46	2.16
Sephadex LH-20	20.14	2.74
Sephadex LH-20 (Re-chromatography)	18.39	3.00
RP-HPLC	10.50	5.25

[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}.

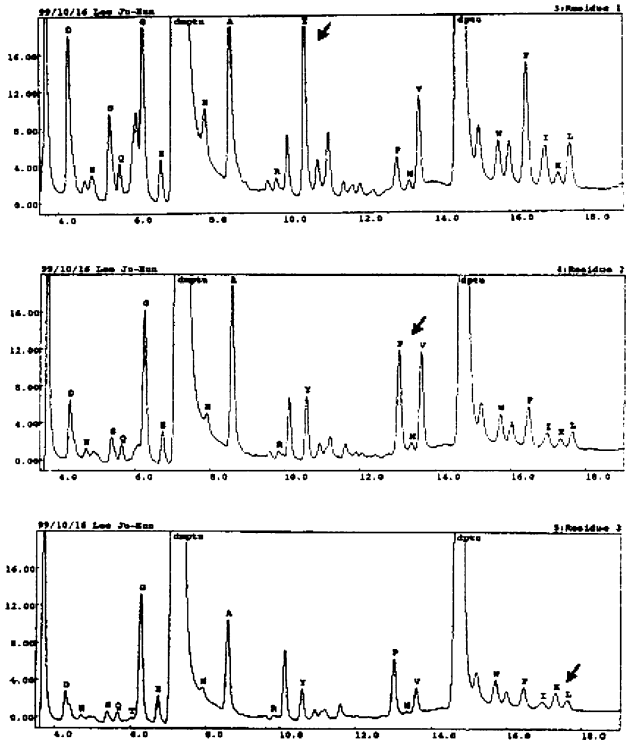

Fig. 6. Peptide sequencing profile of the purified ACE inhibitory peptide, Tyr-Pro-Lys obtained from *Brassica oleracea Italica*.

activity, was revealed to be Tyr-Pro-Lys, having IC_{50} value of 10.5 μg protein/ml. The amino acid sequences from some proteins including mushroom and fish proteins with high ACE inhibitory activities were known to be a tripeptide (Byun & Kim, 2001; Fahmi et al., 2004; Lee et al., 2004). Cheung, Wang, Ondetti, Sabo, and Cushman (1980) reported the results of a series of inhibitory peptides against ACE, indicating that aromatic amino acids at the C-terminal and branched-chain aliphatic amino acids at the N-terminal were suitable for a peptide binding to ACE as a competitive inhibitor. However, other reports have demonstrated that inhibitory peptides possess an aliphatic amino acid residue at their C-terminal. These findings are consistent with

our results that the tripeptide from broccoli has a lysine at its C-terminal. Suetsuna (1998) isolated 7 ACE inhibitory peptides from an aqueous extract of garlic. Most peptides except Phe-Tyr (3.74 μM) had lesser activities than the tripeptide, Tyr-Pro-Lys (23.7 μM) purified in this work. On the other hand, the IC_{50} value of Tyr-Gln-Tyr derived from the buckwheat digest by Li et al. (2002) was 4 μM, which was stronger than that of the peptide (23.7 μM) purified in our study. Megias et al. (2004) reported the IC_{50} value of the octapeptide from sunflower protein was 6.9 μM. However, the amino acid sequence of their peptides and their parents were unique making comparisons difficult. In addition, 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 summary, our results prove that the ACE inhibitory peptides with high activities could be derived from protein of broccoli (*Brassica oleracea Italica*). Therefore, if further studies on animal experiments or clinical trials are performed, these peptides can be used as a possible treatment for hypertension.

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