ISOLATION AND IDENTIFICATION OF ANGIOTENSIN I-CONVERTING ENZYME INHIBITORY PEPTIDES FROM EMZYMATIC PROTEOLYSATE OF PORK LOIN

Kuk-Hwan Seol[1], Dong-In Son[2], Hyun-Jin Kim[2], Thirawong Prayad[3], and Mooha Lee[3]

[1]National Institute of Animal Science, Rural Development Administration, Suwon, 441-706, Republic of Korea, [2]Korea Food Research Institute, Sungnam, 463-746, Republic of Korea, [3]Department of Agricultural Biotechnology & Research Institute for Agriculture and Life Sciences, Seoul National University, Seoul, 151-921, Republic of Korea.

Abstract – This study was performed to identify the angiotensin I-converting enzyme (ACE) inhibitory peptides from pork loin. Large molecules with higher than 3,000 Da were removed by ultra-filtration from crude enzymatic hydrolysates of pork loin. Crude peptide extract from thermolysin injected pork showed the highest ACE inhibitory activity (IC_{50} = 606 μg/mL). It was further purified to select the fraction with the highest ACE inhibitory activity using gel-filtration and RP-HPLC. There were two main fractions (7th and 12th) with high ACE inhibitory activity, and 5 peptides (VFPS, LLGR, LKYP, EPCLAT, and LVGRPRHGQ) were identified by UPLC-Q-TOF-MS/MS system. Peptide LCGRPRHGQ showed the highest ACE inhibitory activity (IC_{50}) of 15.69 μM/mL.

Key Words – ACE inhibitory peptide, thermolysin, UPLC-Q-TOF-MS/MS

I. INTRODUCTION

Nutraceuticals pertain to natural chemical components in food items which have been considered beneficial to the human body, specifically for the prevention, treatment, or improvement of various physiological conditions [1, 2]. Numerous bioactive compounds have now been isolated and characterized [3], including biologically active peptides with immune-modulatory [4], antimicrobial [5], antithrombotic [6], antioxidant [7], and antihypertensive [8], effects from cured meats [9] and other fermented food items. Biologically active peptides are produced during protein hydrolysis by digestive enzymes such as trypsin, chymotrypsin, or pepsin [5]. In addition, bioactive peptides may also be generated by controlled protein hydrolysis, and these peptides, containing only a few amino acid residues, are able to move across the digestive epithelial barrier and reach the blood vessels, enabling their transport to peripheral organs and ultimately imparting their beneficial effects to the entire organism [11]. Jang and Lee [12] and Jang and colleagues [13] reported the inhibitory effects of Hanwoo myofibrillar and sarcoplasmic proteins on angiotensin I-converting enzyme (ACE) and cancer cell proliferation. However, no study has been performed on the bioactivity of porcine skeletal muscle proteins. This study was performed to isolate and identify the angiotensin I-converting enzyme inhibitory peptides derived from pork loin.

II. MATERIALS AND METHODS

Pork loins were purchased at a livestock processing center in Chungcheongbuk-Do, Korea. Based on the report of Jang and Lee [12], loin samples were injected with enzyme protease type XIII (P2143, Sigma, USA) and thermolysin (T7902, Sigma, USA), separately or in combination as shown in Table 1 and stored for 24 h at 5°C for enzymatic proteolysis of muscle proteins. Pork peptide extracts were separated into a high molecular weight fraction and a low molecular weight fraction by ultrafiltration at 4 °C using PM-10 membrane (MWCO, 10,000; Amicon Co., Beverly, MA) and Ultracel 3K membrane (MWCO, 3,000; Amicon Co., Beverly, MA), subsequently lyophilized and kept in a deep-freezer at -70 °C for further use in gel-filtration. Selected gel-filtrated fraction was lyophilized gain and dissolved in water containing 1% trifluoroacetic acid (TFA). Twenty μL of fraction solution was applied to Vydac C18 reverse-phase column (4.5×25cm; 5A, 300μm pore size) for separation. Distilled water containing 0.5% TFA and acetonitrile (ACN) containing 0.1% TFA were used for mobile phase. An UPLC system (Waters) equipped with a binary solvent delivery system, an autosampler, and a photodiode array (PDA) detector was used to analyze peptide. An Acquity

UPLC BEH C18 (2.1 × 100 mm, 1.7 μm; Waters) equipped with the UPLC system was equilibrated with water containing 0.1 % TFA. The HPLC fraction (5 μL) was injected into the column and eluted in a gradient with ACN containing 0.1 % TFA at a flow rate of 0.35 mL/min for 12 min. The absorbance of the eluent was detected at 214 nm by a PDA detector and a Quadrupole-Time of Flight (Q-TOF) mass spectrometry. Peptides separated by C18-UPLC were analyzed and assigned by Q-TOF mass spectrometry (Waters) equipped with the UPLC system. The Q-TOF was operated in ESI positive mode. The capillary and sampling cone voltages were set at 2.78 kV and 26 V, respectively. The desolvation flow was set to 700 L/h at a temperature of 300°C and the source temperature was set to 100°C. The TOF MS data was collected in the range of *m/z* 50-1,000 with a scan time of 0.2 sec and interscan delaytime of 0.02 sec. The MS/MS spectra of peptides were obtained by a collision energy ramp from 10-45 eV. The accurate mass and composition for the precursor ions and the fragment ions were calculated and sequenced by MassLynx (Waters) incorporated in the instrument. Peptides were analyzed and sequenced by a peptide sequencer in BioLynx (Waters) incorporated in the instrument.

Angiotensin converting enzyme (ACE, from rabbit lung) and hippuryl-L-histidyl-L-leucine (HHL) were purchased from Sigma (St. Louis, MO, USA) and all other chemicals used were analytical grade (Fisher, Springfield, NJ). The determination of ACE inhibitory activity was performed by the spectrophotometric method described by Cushman and Cheung (1971). The IC_{50} value, defined as the concentration of a peptide that inhibits 50 % of the ACE activity, was determined by measuring the ACE inhibitory activity and peptide contents of each extracts after regression analysis.

Whole experiments were replicated 3 times with 2 observations per each replication. Statistical analysis was performed with the SAS program for Window V9.1 (SAS Institute, Cary, NC, USA). General linear model (GLM) with Duncan's multiple range test was carried out to analyze the significant differences among the treatments ($P < 0.05$).

Table 1. Preparation of Pork *M. Longissimus* with enzyme injection

Sample	Treatment
Fresh	Fresh pork
Control	No enzyme injected
Enzyme 1	100 ppm of protease type XIII injected
Enzyme 2	80 ppm of thermolysin injected
Enzyme 3	100 ppm of protease type XIII + 80 ppm of thermolysin injected

III. RESULTS AND DISCUSSION

The ACE inhibition rates of crude peptide extracts (less than 3,000 Da) were shown in Figure 1. Extracts from thermolysin injected (Enzyme 2, 85%) and protease type XIII and thermolysin injected pork (Enzyme 3, 90%) were significantly higher than others ($P < 0.05$). The crude extract of Enzyme 2 showed the highest ACE inhibitory activity (IC_{50} = 606 μg/mL), and that of Enzyme 3, Enzyme 1, and Control was 687, 108,576, and 26,645 μg/mL, respectively. Therefore, the crude peptide extract from Enzyme 2 was selected for further analysis and isolation.

The crude extract was adapted to the gel-filtration system and there were 4 major fraction groups (Fig. 2). Especially, fraction group III showed the highest ACE inhibition rate (79%). That fraction was lyophilized and adapted to RP-HPLC and further separation and analysis were carried out.

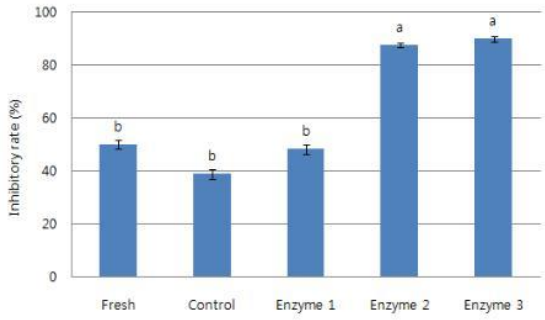

Figure 1. The ACE inhibition rate of crude peptide extracts derived from pork loin

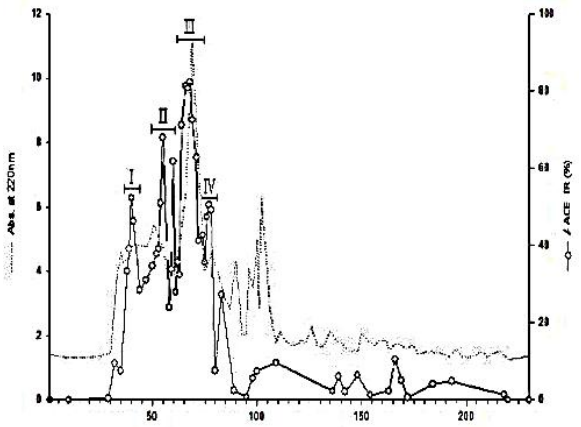

Figure 2. The absorbance at 220 nm and ACE inhibition rate of gel-filtration fractions derived from thermolysin injected pork loin

The chromatogram and ACE inhibition rate of Group III fraction are shown in Figure 3 and 17 peak fractions were separated depending on their ACE inhibition rate. The 7th and 12th fraction showed high ACE inhibition rate of 89 and 79 %, respectively. The most potent fractions which showed high ACE inhibition rate were analyzed by LC-MS/MS to identify the amino acid sequences.

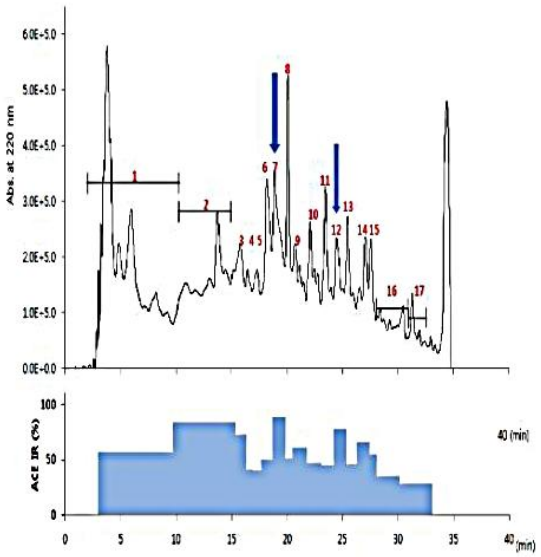

Figure 3. The chromatogram and ACE inhibition rate of RP-HPLC fractions of Group III fractions

According to the MS/MS spectrum, all the peptides were identified by *de novo* sequencing. The total mass of the peptide combination was obtained from the mass detector, and then the monoisotopic mass of an individual amino acid was subtracted to identify the exact sequence with an accurate mass. MS/MS spectra of potent peptides were displayed using a single positively charged ion ($M + [H]^+$). The BioLynx identified 5 candidate peptides and they were LVGRPRHGQ (Fig. 4) and VFPS from the 7th fraction, and LLGR, LKYP, and EPCLAT from the 12th fraction, respectively. These peptides were chemically synthesized by Peptron Ltd. (Daejeon, Korea) using Peptr EX^{TM} automatic peptide synthesizer for further analysis of ACE inhibitory activity (IC_{50}).

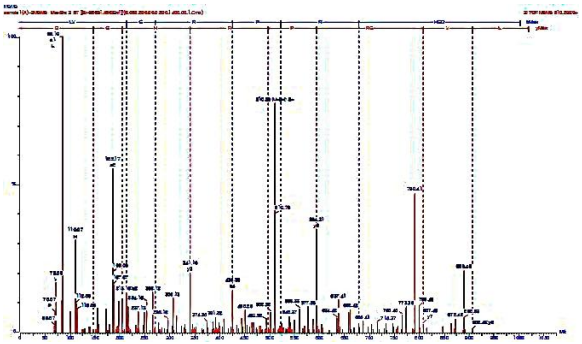

Figure 4. Identification of ACE inhibitory peptide LVGRPRHGQ derived from thermolysin injected pork using Q-TOF-MS/MS spectrum

The ACE inhibitory activities (IC_{50}) of chemically synthesized 5 candidate peptides were measured (Table 2) and the nonapeptide, LVGRPRHGQ, exhibited the highest ACE inhibitory activity (the lowest IC_{50}) of 15.69 μM/mL.

When the bioactive peptides meet gastric enzymes their activities in the body may also be affected. Jang and colleagues [14] identified 4 peptides which contained below 8 amino acids and digested them by gastrointestinal (GI) enzymes such as pepsin, trypsin, and α-chymotrypsin. They showed that no significant change of ACE inhibition activity was observed. However, further study needs to determine if this peptide LVGRPRHGQ will survive in the GI digestive system and show anti-hypertensive effect in vivo.

Table 2. The ACE inhibitory activity of chemically synthesized peptides

Peptides	Molecular weight (Da)	IC_{50} value (μM)
VFPS	449.2	3,606
LLGR	458.3	240.02
LKYP	520.3	641.9
EPCLAT	633.3	173.7
LVGRPRHGQ	1019.6	15.69
Captopril	217.29	0.017

IV. CONCLUSION

Several active fractions with ACE inhibition were obtained from enzymatic proteolysis of pork loin. The most bioactive peptide fraction was resulted from the hydrolysis of porcine muscular proteins with Thermolysin alone. Further purification resulted in a nonapeptide which exhibited the highest ACE inhibitory effect. Further studies are necessary to examine if this peptide will survive the GI digestion in vivo and has other bioactivities.

REFERENCES

1. Wildman, R. E. C. (2000a). Nutraceuticals: A brief review of historical and teleological aspects. In R. E. C., Wildman (Ed.), Handbook of nutraceuticals and functional foods (pp. 1-12). Florida: CRC Press.
2. Wildman, R. E. C. (2000b). Classifying nutraceuticals. In R. E. C., Wildman (Ed.), Handbook of nutraceuticals and functional foods (pp. 1-12). Florida: CRC Press.
3. Hasler, C. M. (1998). Functional foods: Their role in disease prevention and health promotion. Food Technology, 52, 63-70.
4. Clare, D. A., & Swaisgood, H. E. (2000). Bioactive milk peptides: a prospectus. Journal of Dairy Science, 83, 1187-1195.
5. Liepke, C., Zucht, H. D., Forssmann, W. G., & Standker, L. (2001). Purification of novel peptide antibiotics from human milk. Journal of Chromatography B, 752, 369-377.
6. Fiat, A. M., Migliore-Samour, D., Jolles, P., Drouet, L., Sollier, C. B. D., & Cean, J. (1993). Biologically active peptides from milk proteins with emphasis on two examples concerning antithrombotic and immunomodulating activities. Journal of Dairy Science, 76, 301-310.
7. Kim, S. K., Lee, H. C., Byun, H. G., & Jeon, Y. J. (1996). Isolation and characterization of antioxidative peptides from enzymatic hydrolysates of yellowfin sole skin gelation. Journal of Korean Fishery Society, 29, 246-255.
8. Ariyoshi, Y. (1993). Angiotensin-converting enzyme inhibitors derived from food proteins. Trends of Food Science Technology, 4, 139-144.
9. Rodriguez-Nunez, E., Aristoy, M., & Toldra, G. (1995). Peptide generation in the processing of dry-cured ham. Food Chemistry, 53, 187-190.
10. Yamamoto, N., Maeno, M., & Takano, T. (1999). Purification and characterization of an antihypertensive peptide from a yogurt-like product fermented by Lactobacillus helveticus CPN4. Journal of Dairy Science, 82, 1388-1393.
11. Yust, M. M., Pedroche, J., Giron-Calle, J., Alaiz, M., Millan, F., & Vioque, J. (2003). Production of ACE inhibitory peptides by digestion of chickpea legumin with alcalase. Food Chemistry, 81, 363-369.
12. Jang, A., & Lee, M. (2005). Purification and identification of angiotensin converting enzyme inhibitory peptides from beef hydrolysates. Meat Science, 69, 653-661.
13. Jang, A., Jo, C., Kang, K. S., & Lee, M. (2008). Antimicrobial and human cancer cell cytotoxic effect of synthetic angiotensin-converting enzyme (ACE) inhibitory peptides. Food Chemistry, 107, 327-336.
14. Jang, A., Jo, C., & Lee, M. (2007). Storage stability of the synthetic angiotensin converting enzyme (ACE) inhibitory peptides separated from beef sarcoplasmic protein extracts at different pH, temperature, and gastric digestion. Food Science and Biotechnology, 16(4), 572-575.