Korean J. Food Sci. Ani. Resour.
Vol. 31, No. 5, pp. 663~667(2011)
DOI http://dx.do.org/10.5851/kosfa.2011.31.5.663

ARTICLE

Assessment of the Inhibitory Activity of Peptide Extracts from Hanwoo *Musculus Longissimus* on Angiotensin I-Converting Enzyme

Kuk-Hwan Seol[1], Ji-Hye Song[2], Thirawong Prayad, Hyoun Wook Kim[1], Aera Jang[1], Jun-Sang Ham[1], Mi-Hwa Oh[1], Dong-Hun Kim[1], and Mooha Lee*

Department of Animal and Food Biotechnology, Research Institute for Agriculture and Life Science, Seoul National University, Seoul 151-921, Korea

[1]*National Institute of Animal Science, Rural Development Administration, Suwon 441-706, Korea*

[2]*CJ Cheiljedang Corporation Foods R&D Nutraceuticals & Functional Foods Center, Seoul 152-051, Korea*

Abstract

This study was performed to measure the angiotensin I-converting enzyme (ACE) inhibitory activity of peptide extracts derived from the enzymatic proteolysis of Hanwoo *Musculus longissimus* (*M. longissimus*) during cold storage. Thermolysin (80 ppm, w/w) and protease type XIII (100 ppm, w/w) were injected separately or in combination for the enzymatic proteolysis of sarcoplasmic and myofibrillar proteins prior to storage at 5°C (T1) or at -1°C (T2) in a chilling room for 9 days. Beef injected with thermolysin (E2) and thermolysin+protease type XIII (E3) showed a significantly higher degree of hydrolysis at both storage temperatures ($p<0.05$). During the storage period, T1E2 at day 6 and T1E3 at day 9 showed the strongest ACE inhibitory activity with sarcoplasmic and myofibrillar protein proteolysates. Macromolecules greater than 10,000 Da were removed by ultra filtration, and the filtrates were separated into fractions using gel filtration. Five and three major fractions were collected from S-T1E2-6 and M-T1E3-9 extracts, respectively, and the 4th fraction of the S-T1E2-6 extracts showed the highest ACE inhibitory rate of 61.96±7.41%.

Key words: angiotensin I-converting enzyme inhibitory activity, Hanwoo beef, thermolysin, protease type XIII

Introduction

The renin-angiotensin system is one of the most important humoral vasoconstrictor and vasodilator mechanisms involved in blood pressure regulation. In this system, angiotensinogen is first converted into a prehypertensive hormone angiotensin I (DRVYIHPE-HL), through the action of rennin, which is secreted by the kidneys. Angiotensin I is further converted to angiotensin II (DRVYIHPE), the active form of the hormone, by the action of angiotensin I-converting enzyme (ACE). The ACE catalyzes the production of the vasoconstrictor angiotensin II as well as the inactivation of the vasodilator bradykinin (RPPGFSPFR) or encephalitis (Ariyoshi, 1993; Hazato and Kase, 1986; Maruyama *et al*., 1985).

Inhibition of ACE is an established therapy for hypertension (Vane, 1999). Bioactive peptides are most commonly produced by enzymatic hydrolysis of whole molecules. Peptides capable of inhibiting ACE have recently been identified in the tryptic hydrolysates of bovine α_{s2}-casein (Tauzin *et al*., 2002) and bovine, ovine and caprine κ-casein macropeptides (Manso and López-Fandino, 2003). Different digestive enzyme combinations of proteinases including alcalase, chymotrypsin, pancreatin, pepsin and thermolysin as well as enzymes from bacterial and fungal sources have been used to generate bioactive peptides from various proteins (Kilara and Panyam, 2003; Korhonen and Pihlanto, 2003).

In this study, we examined the activity of peptides derived from Hanwoo *M. longissimuss* that are capable of inhibiting ACE under several enzymatic treatments and storage conditions.

Materials and Methods

Praparation of crude peptide extracts from Hanwoo *M. longissimus*

M. longissimus from Hanwoo steers aged 28-30 mon were collected from Gangwon Livestock Process Center

*Corresponding author: Mooha Lee, Department of Animal and Food Biotechnology, Research Institute for Agriculture and Life Science, Seoul National University, Seoul 151-921, Korea. Tel: 82-2-880-4820, Fax: 82-2-873-4804, E-mail: moohalee@snu.ac.kr

(Gangwon LPC, Korea) after slaughter. A study by Jang and Lee (2005) showed that thermolysin and protease type XIII had high proteolytic activities on muscular sarcoplasmic and myofibrillar proteins, therefore, on the basis of this study, thermolysin (T7902, Sigma, USA) and protease type XIII (P2143, Sigma, USA) were injected in the beef separately or in combination. The beef was then stored at 5°C (T1) or -1°C (T2) in chilling room for 9 d as shown in Table 1. On the 3rd, 6th and 9th d of storage, the sarcoplasmic and myofibrillar proteins were extracted from the beef, and high molecular peptides of greater than 10,000 Da were removed using an ultra-filtration system at 4°C with a PM-10 membrane (MWCO, 10,000 Da; Amicon Co., USA). After filtration, the filtrates were frozen in a deep freezer and then used to measure the degree of hydrolysis, and ACE inhibitory activity. Subsequently, these filtrates were further purified by gel filtration.

Reagents

ACE (from rabbit lung) and hippuryl-L-histidyl-L-leucine (HHL) were purchased from Sigma (USA) and all other chemicals used were of analytical grade (Fisher, USA).

Extraction of muscular protein hydrolyzates

Hanwoo muscular protein proteolysates were extracted by the modified method described in the study by Jang and Lee (2005). Beef samples were homogenized for 3 min in 50 mL of 20 mM phosphate buffer (pH 7.4). Then homogenates were centrifuged at 4°C, 10,000 g for 20 min and filter the supernatant with a 0.45 μm membrane filter, and used as sarcoplasmic protein proteolysate extracts. Myofibrillar protein proteolysate extracts were separated from the pellets by further extraction. Fifty milliliters of 20 mM phosphate buffer (pH 7.4) and 50 mL of 0.1% Triton X-100 were added to the pellets. The mixtures were homogenized for 2 min and centrifuged at 4°C at 10,000 g for 20 min, and the pellets were mixed with 50 mL of 0.1 M phosphate buffer and 50 mL of 1.1 M KI and homogenized for 2 min. The homogenates were then centrifuged at 4°C at 10,000 g for 20 min. The supernatants were filtered with a 0.45-μm membrane filter to separate the myofibrillar protein proteolysate extracts. Both the sarcoplasmic and myofibrillar protein proteolysate extracts were heated at 90°C in a water bath for 15 min to inactivate the injected enzymes.

Table 1. Preparation of Hanwoo *M. longissimus* with various enzymes and storage conditions

	T1	T2
Con	No enzyme	No enzyme
E1	80 ppm protease type XIII	80 ppm protease type XIII
E2	100 ppm thermolysin	100 ppm thermolysin
E3	80 ppm protease type XIII + 100 ppm thermolysin	80 ppm protease type XIII + 100 ppm thermolysin

T1, 5°C chilling room; T2, -1°C chilling room
Con, no enzyme; E1, protease type XIII 100 ppm/meat sample; E2, thermolysin 80 ppm/meat sample; E3, protease type XIII 100 ppm/meat sample + thermolysin 80 ppm/meat sample

Analysis of protein content and the degree of hydrolysis

The total amount of protein in the extracts was measured using a protein assay kit (Sigma Co., USA) and bovine serum albumin was used as the standard. The degree of hydrolysis was analyzed by measuring the amount of nitrogen dissolved in 10% trichloroacetic acid. Peptide extracts were mixed with an equal volume of 20% trichloroacetic acid, reacted for 30 min at room temperature, and then centrifuged at 1,750 g for 10 min. Soluble nitrogen was measured using protein assay kit (Sigma Co., USA). The degree of hydrolysis was calculated as follows:

$$\% \text{ DH} = (10\% \text{ TCA-soluble protein/total protein}) \times 100$$

ACE inhibitory activity

The ACE inhibitory activity was determined using the spectrophotometric method described by Cushman and Cheung (1971). For each assay, 100 μL of HHL (12.5 mM in 0.05 M sodium borate buffer) were incubated at 37°C for 5 min. After incubation, 50 μL of bovine peptide extracts and 150 μL of ACE (peptidyldipeptide hydrolase, from rabbit lung acetone extract) were added, and the mixture incubated for 1 h. The enzymatic reaction was stopped by adding 250 μL of 0.5 N HCl. The hippuric acid formed by the action of the ACE on HHL was extracted from the acidified solution into 1 mL ethyl acetate by vortex mixing for 15 s. This mixture then was centrifuged at 3,290 g for 10 min at 4°C, and a 0.7 mL aliquot of each ethyl acetate layer was transferred to clean tubes and evaporated by heating at 95°C for 20 min in a water bath. The hippuric acid was dissolved again in 3 mL of 1 M NaCl, and the amount formed was determined by measuring the absorbance at 228 nm. 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

extract after regression analysis.

$$\text{ACE inhibitory activity} = \left(1 - \frac{S - S.C.}{B - B.C.}\right) \times 100$$

S, OD value of sample; S.C., OD value of sample control; B, OD value of blank; B.C., OD value of blank control

Separation of the active fraction inhibiting ACE

Peptide extracts that showed high ACE inhibitory activity were selected. Molecules greater than 10,000 Da were removed by ultra filtration at 4°C by using a PM-10 membrane, and the ACE inhibitory activity was measured. The filtrate was refiltered using a 0.45-μm Millipore membrane filter and loaded on the XK 2.6 mm×1 m (Amersham Pharmacia Biotech., UK) gel-filtration column filled with Sephadex® G-25 resins (GE Healthcare Biosciences, USA). Phosphate buffer (20 mM, pH 7.0) containing 0.05% (w/v) sodium azide was used for the mobile phase, and the flow rate was 5.0 mL/min. Each fraction was collected and measured for ACE inhibitory activity.

Statistical analysis

All analyses were performed in triplicate. Statistical analysis was performed with the SAS program for Windows V9.1 (SAS Institute, USA). Two-way ANOVA was carried out with Duncan's multiple range test to analyze the significant differences among the treatments ($p<0.05$).

Results and Discussion

Degrees of proteolysis

The degrees of proteolysis of the sarcoplasmic protein proteolysate (SPP) and myofibrillar protein proteolysate (MPP) extracted from samples stored at 5°C or -1°C during the storage period are shown in Tables 2 and 3. Enzymatically proteolyzed E2 and E3 samples stored at both temperature (T1 and T2) showed significantly higher degree of proteolysis in sarcoplasmic and myofibrillar protein proteolysate extracts than others (Con and E1) ($p<0.05$). Furthermore, the degree of proteolysis of extracts stored at 5°C in a chilling room was higher than those of samples stored at -1°C. On the 9th day of storage, the degree of proteolysis of E3, injected with both thermolysin and protease type XIII, was high.

Although the degree of proteolysis of the Hanwoo muscular protein was lower than that previously reported by of Jang *et al.* (2003), who carried out proteolysis at the optimum temperature of enzyme and reported almost 70% after 24 h of reaction, it gradually increased during the storage period. These results are similar to those of Lee *et al*. (2005), which showed that the degree of proteolysis of every treated enzyme increases during storage. These results also correspond with those of Jang *et al*. (2003), which showed that the degree of proteolysis was higher in the samples injected thermolysin + protease type III, thermolysin, and protease type XIII, with the degree increasing in that order.

Table 2. The degree of proteolysis of sarcoplasmic proteins with several proteases during storage

	3 d	6 d	9 d
T1Con	2.98±1.44^{e}	2.58±1.96^{c}	4.58±2.27^{cd}
T1E1	5.60±0.31^{cdeB}	5.27±1.27^{cB}	7.98±1.31^{cA}
T1E2	9.71±0.46^{bcB}	16.46±5.36^{abA}	15.69±1.26^{bAB}
T1E3	12.08±0.83^{abB}	12.87±3.65^{abB}	19.91±0.19^{aA}
T2Con	3.49±0.93^{bde}	2.79±0.39^{c}	3.85±1.38^{d}
T2E1	7.90±6.32^{bcd}	6.10±0.93^{c}	5.36±0.42^{cd}
T2E2	15.40±1.60^{a}	16.90±2.80^{a}	15.84±3.28^{b}
T2E3	12.60±2.40^{abB}	11.84±0.96^{bB}	17.85±3.23^{abA}

Values are expressed as mean±SD.
T1, 5°C chilling room; T2, -1°C chilling room.
Con, no enzyme; E1, protease type XIII 100 ppm/meat sample; E2, thermolysin 80 ppm/meat sample; E3, protease type XIII 100 ppm/meat sample + thermolysin 80 ppm/meat sample.
[a-e]Means in the same column with different letters are significantly different ($p< 0.05$).
[A,B]Means in the same row with different letters are significantly different ($p < 0.05$).

Table 3. The degree of proteolysis of myofibrillar proteins with several proteases during storage

	3 d	6 d	9 d
T1Con	2.30±0.65^{abA}	0.80±0.14^{bB}	2.96±0.33^{bA}
T1E1	3.59±0.30^{ab}	2.56±1.20^{ab}	3.56±0.96^{ab}
T1E2	4.22±0.91^{ab}	4.01±0.04^{a}	3.99±1.04^{ab}
T1E3	3.89±1.80^{ab}	3.00±0.79^{ab}	4.26±0.21^{ab}
T2Con	1.14±0.13^{b}	3.93±2.16^{a}	4.77±3.13^{ab}
T2E1	3.14±2.50^{ab}	2.16±0.25^{ab}	5.04±1.3^{ab}
T2E2	4.75±0.95^{a}	4.81±1.72^{a}	6.59±0.02^{ab}
T2E3	4.36±3.12^{a}	4.93±3.40^{a}	6.98±3.89^{a}

Values are expressed as mean±SD.
T1, 5°C chilling room; T2, -1°C chilling room.
Con, no enzyme; E1, protease type XIII 100 ppm/meat sample; E2, thermolysin 80 ppm/meat sample; E3, protease type XIII 100 ppm/meat sample + thermolysin 80 ppm/meat sample.
[a,b]Means in the same column with different letters are significantly different ($p < 0.05$).
[A,B]Means in the same row with different letters are significantly different ($p < 0.05$).

ACE inhibitory activity

Fig. 1 and 2 show the ACE inhibitory activities of SPP

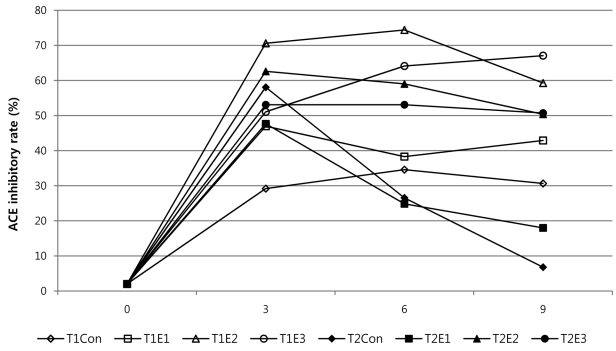

Fig. 1. The ACE inhibitory activities of sarcoplasmic protein proteolysates derived from Hanwoo *M. longissimus* with several proteases during storage at -1 and 5°C. T1, 5°C chilling room; T2, -1°C chilling room; Con, no enzyme; E1, protease type XIII 100 ppm/meat sample; E2, thermolysin 80 ppm/meat sample; E3, protease type XIII 100 ppm/meat sample + thermolysin 80 ppm/meat sample.

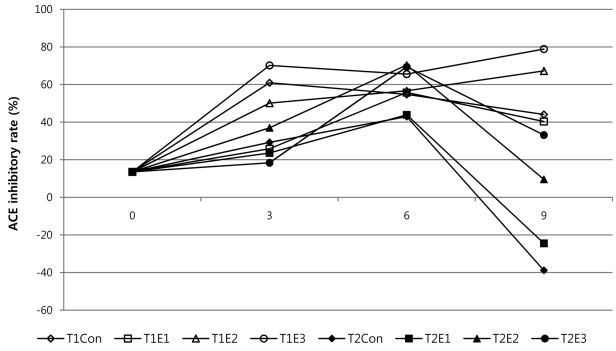

Fig. 2. The ACE inhibitory activities of myofibrillar protein proteolysates derived from Hanwoo *M. longissimus* with several proteases during storage at -1 and 5°C. T1, 5°C chilling room; T2, -1°C chilling room; Con, no enzyme; E1, protease type XIII 100 ppm/meat sample; E2, thermolysin 80 ppm/meat sample; E3, protease type XIII 100 ppm/meat sample + thermolysin 80 ppm/meat sample.

and MPP extracted from enzyme-injected Hanwoo muscles. Although, the extract at day 0 showed ACE inhibitory activity of less than 20%, most extracts showed increased ACE inhibitory activities as storage period increased. However, some extracts showed decreased activity at day 9. Among the SPP extracts, those obtained from thermolysin-injected beef stored for 6 d at 5°C (S-T1-E2-6) showed the highest ACE inhibitory activity (74.39±6.88%). On the contrary, among the MPP extracts, protease type XIII- and thermolysin-injected beef stored for 9 d at 5°C (M-T1-E3-9) showed the highest ACE inhibitory activities (78.89±3.79%). The ACE inhibitory activity of MPP extracted from beef stored at 5°C (T1) was slightly higher than that of proteolysates extracted from beef stored at -1°C (T2), and this result corresponded with the degree of hydrolysis, indicating that the ACE inhibitory activity depends on the degree of hydrolysis. Most extracts derived from enzyme-injected beef showed similar or higher ACE inhibitory activities than extracts obtained from control beef; this shows that enzymatic hydrolysis of muscular proteins may generate peptides capable of inhibiting ACE inhibitory in beef.

Previous studies (Jang *et al*., 2003; Lee *et al*., 2005) showed that the ACE inhibitory activity of peptides produced by enzymatic hydrolysis was decreased after a specific reaction time. Although a relationship exists between the degree of hydrolysis and ACE inhibitory activity, it does not always increase. A specific amino acid sequence that can bind to the ACE active site may exist, but if this peptide is degraded too much, it would lose its inhibitory activity (Lee *et al*., 2005).

ACE inhibitory activities of ultra-filtrated and gel-filtrated fractions

Extracts S-T1-E2-6 of SPP and M-T1-E3-9 of MPP, which showed the highest ACE inhibitory activity in each protein proteolysate group, were fractionated by morecular weight using ultra filtration with a PM-10 membrane filter. The filtered peptide fractions of less than 10,000 Da were lyophilized and analyzed for ACE inhibitory activity again (Table 4), and then they were used for further isolation using gel filtration. The ACE inhibitory activities of S-T1-E2-6 and M-T1-E3-9 were 77.12±0.27% and 80.19±0.95%, respectively, and slightly higher than the activities before ultra filtration. This shows that ACE inhibitory activity was considerably contributed by small peptides; this result corresponds to that of previous study by Jang and Lee (2005) and other studies (Astawan *et al*., 1995; Gomez-Ruiz *et al*., 2002) that reported highest inhibitory activity at around 3,000 Da. By using gel filtration, S-T1-E2-6 and M-T1-E3-9 were separated into 5

Table 4. The ACE inhibitory activities of ultra-filtrated fractions of W-T1-E2-6 and S-T1-E3-9

	S-T1-E2-6	M-T1-E3-9
ACE inhibition activity (%)	77.12±0.27[b]	80.19±0.95[a]

Values are expressed as mean±SD.
S, sarcoplasmic protein proteolysate; M, myofibrillar protein proteolysate
T1, 5°C refrigerator; E2, thermolysin 80 ppm/meat sample, E3, protease type XIII 100 ppm/meat sample + thermolysin 80 ppm/meat sample
[ab]Means in the same column with different letters are significantly different (p<0.05).

Table 5. The ACE inhibitory activities of gel-filtrated fractions from W-T1-E2-6 and S-T1-E3-9

Extract Fraction No.	W-T1-E2-6	S-T1-E3-9
1	4.15±2.79	52.57±1.53
2	34.39±0.28	28.86±3.64
3	44.07±3.35	30.89±2.68
4	61.96±7.41	-
5	43.08±1.68	-

Values are expressed as mean±SD.
S, sarcoplasmic protein proteolysate; M, myofibrillar protein proteolysate
T1, 5°C refrigerator; E2, thermolysin 80 ppm/meat sample; E3, protease type XIII 100 ppm/meat sample + thermolysin 80 ppm/meat sample

and 3 fractions, respectively (data not shown). The ACE inhibitory activities of those fractions are shown in Table 5. The 4th fraction at S-T1-E2-6 showed the highest ACE inhibitory activity of 61.96±7.41%, and the 1st fraction of M-T1-E3-9 showed 52.57±1.53%. In this study, we did not analyze the concentration of these fractions, and separated peptides need to be identified in future studies.

The enzymatic hydrolysis of Hanwoo muscular protein may generate antihypertensive peptides. Although we did not identify the active peptides in this study, the existence of active fractions was confirmed. If significant amounts of these peptides could be generated during meat aging or manufacturing of meat products, this research would be a scientific basis for a novel industrial technology. However, further studies are needed to identify the antihypertensive peptides and other bioactive peptides derived from Hanwoo beef.

References

1. Ariyoshi, Y. (1993) Angiotensin-converting enzyme inhibitors derived from food proteins. *Trends Food Sci. Technol*. **4**, 139-144.
2. Astawan, M., Wahyuni, M., Yasuhara, T., Yamada, K., Tadokoro, T., and Maekawa, A. (1995) Effects of angiotensin I-converting enzyme inhibitory substances derived from Indonesian dried-salted fish on blood pressure of rats. *Biosci. Biotech. Bioch*. **59**, 425-429.
3. Cushman, D. W. and Cheung, H. S. (1971) Spectrophotometric assay and properties of the angiotensin-converting enzyme of rabbit lung. *Biochem. Pharmacol*. **20**, 1637-1638.
4. Gomez-Ruiz, J. A., Ramos, M., and Recio, I. (2002) Angiotesin converting enzyme-inhibitory peptides in Manchego cheeses manufactured with different starters cultures. *Int. Dairy J*. **12**, 697-706.
5. Hazato, T. and Kase R. (1986) Isolation of angiotensin-I converting enzyme inhibitor from porcine plasma. *Biochem. Biophys. Res. Comm*. **139**, 52-55.
6. Jang A. and Lee M. (2005) Purification and identification of angiotensin converting enzyme inhibitory peptides from beef hydrolysates. *Meat Sci*. **69**, 653-661.
7. Jang S. H., Jang A., Kim K. J., Cheon Y. H., Min J. S., Lee S. O., and Lee M. (2003). Purification and isolation for antihypertensive peptides from beef heart and spleen. *J. Ani. Sci. Tech*. **45**, 319-326.
8. Kilara, A. and Panyam, D. (2003) Peptides from milk proteins and their properties. *Crit. Rev. Food Sci. Nutr*. **43**, 607-633.
9. Korhonen H. and Pihlanto A. (2003). Food-derived bioactive peptides - opportunities for designing future foods. *Curr. Pharm. Des*. **9**, 1297-1308.
10. Lee K. J., Kim S. B., Ryu J. S., Shin H. S., and Lim J. W. (2005). Separation and purification of angiotensin converting enzyme inhibitory peptides derived from goat's milk whey hydrolysates. *Korean J. Ani. Sci. Tech*. **47**, 83-90.
11. Manso, M. A. and López-Fandino, R. (2003) Angiotensin I converting enzyme-inhibitory activity of bovine; ovine; and caprine kappa-casein macropeptides and their tryptic hydrolysates. *J. Food Protec*. **66**, 1686-1692.
12. Maruyama, S., Nakagomi, K., Tomizuka, N., and Suzuki, H. (1985) Angiotensin-I converting enzyme inhibitor derived from an enzymatic hydrolysate of casein. II. Isolation and dradykinin-potentiating activity in the uterus and ileum of rats. *Agric. Biol. Chem*. **49**, 1405-1409.
13. Tauzin, J., Miclo, L., and Gaillard, J. (2002) Angiotensin I-converting enzyme inhibitory peptides from tryptic hydrolysate of bovine α_{s2}-casein. *FEBS Lett*. **531**, 369-374.
14. Vane, J. R. (1999) The history of inhibitors of angiotensin converting enzyme. *J. Physiol. Pharmacol*. **50**, 489-498.

(Received 2010.11.29/Revised 2011.9.6/Accepted 2011.9.16)