Production of Angiotensin-I Converting Enzyme Inhibitory Hydrolysates from Egg Albumen

H. S. Kim*, J. S. Ham, S. G. Jeong, Y. M. Yoo, H. S. Chae, C. N. Ahn and J. M. Lee

Products and Utilization Division, National Livestock Research Institute, Suwon 441-350, Korea

ABSTRACT : ACE (Angiotensin-I converting enzyme) inhibitory peptides derived from foods are thought to suppress high blood pressure by inhibiting ACE. We tried to make efficient production of the ACE inhibitory hydrolysate from egg albumen. A hydrolysate digested by neutrase presented the highest ACE inhibitory activity (IC_{50} value=256.35 µg/ml) and the proper proteolysis was occurred by 1.0% enzyme addition and 4 h incubation at 47°C. Antihypertensive effect of neutrase hydrolysate was investigated in spontaneously hypertensive rats (SHR, n=5). Systolic blood pressure (SBP) was decrease by 6.88% (-14.14 mmHg, $p<0.05$) at 3 h after oral administration of 300 mg/kg body weight, and by 13.33% (-27.72 mmHg, $p<0.05$) by emulsified hydrolysate. These results showed that it is very effective to utilize egg albumen as a protein source for the production of ACE inhibitory peptides. However, further studies are required to investigate the methods to increase recovery yield and the isolation of active peptide is necessary for determining its sequence responsible for ACE inhibitory activity. ***(Asian-Aust. J. Anim. Sci. 2003. Vol 16, No. 9 : 1369-1373)***

INTRODUCTION

Hypertention is related to the incidence of coronary heart disease and over 90% of hypertension is known as an essential hypertension. Angiotensin-I converting enzyme (ACE) produces a hypertensive peptide hormone angiotensin-II from angiotensin-I that has an important role in hypertension (Peart, 1976).

Since ACE inhibitory peptide was first obtained from snake venom (Feria, 1965), various ACE inhibitors have been discovered from enzymatic hydrolysates of several food proteins (Maruyama and Suzuki, 1982; Miyosh et al., 1991; Kinoshita et al., 1993; Mullally et al., 1997). Egg albumen, which contains 90% of proteins on dry matter, is the good source of high quality protein and can be obtained with relatively low price. In Korea, though almost of egg albumen is allowed to be discarded, a small portion has been utilized in the production of boiled fish paste and the isolation of lysozyme.

Thus the loss of this valuable protein source should be prevented and we tried to estimate the production of ACE inhibitory hydrolysates from egg albumen.

The aim of this study was to produce egg albumen hydrolysate with ACE inhibitory activity and to identify its antihypertensive effect *in vivo*.

MATERIALS AND METHODS

Enzymatic hydrolysis of egg albumen

Egg albumen powder (Foodpia Co. Korea) was dissolved in distilled water (2%, w/v, pH 7.0) and heated at 85°C for 20 min. After cooling, it was hydrolyzed with alcalase, protamax, flavourzyme, neutrase (Novo Nordisk, Denmark), and promod 192 (Biocatalysts U. K), at 47°C for 24 h (ratio of protein substrate to enzyme, 100:1). The enzymatic reaction was, after hydrolyzation, interrupted by heating at 85°C for 30 min. Insoluble solids were removed by centrifugation (8,000×g for 20 min). Egg albumen hydrolysates were stored at -20°C until assay.

Assay for ACE inhibitory activity

ACE inhibitory activity was determined by the method described in Cushman and Cheung (1971). Briefly, ACE was prepared from ACE acetone powder (Sigma, USA) combined with 4 times of 0.1 M sodium borate buffer (pH 8.3) containing 0.3 M NaCl, stirring overnight at 4°C and centrifugation (8,000×g for 30 min).

Each 5 µl of sample solution (20 mg/ml) was preincubated with 12.5 mM hippuryl-l-histidyl-L-leucine (Hip-His-Leu, Sigma, USA) dissolved in 0.1 M sodium borate buffer (pH 8.3) at 37°C for 5 min. One hundred fifty microliter (µl) of ACE was added for incubation at 37°C for 1 h and the liberated hippuric acid was extracted with ethyl acetate (Sigma, USA). ACE inhibition value (%) was calculated by measuring the absorbances at 228 nm. IC_{50} value was defined as the concentration of peptide (µg/ml) required to reduce 50% of the inhibition ACE activity.

Molecular mass distribution

Molecular mass distributions of the hydrolysates were determined by gel permeation chromatography. The TSK gel G3000 PWXL (7.8 mm×30 cm, Tosho) was fitted for HPLC system (Jasco, Japan). The mobile phase consisted of

Table 1. Recovery yields, IC_{50} values and molecular weight distribution of egg albumen hydrolysates

Enzymes	Recovery yields (%)	IC_{50} value (µg/ml) [1]	Molecular mass distribution (%) [2]			
			≥3,000 Da	3,000-1,000 Da	≤1,000 Da	Average M. W.
Summizyme LP	36.18	331.25±5.24	5.97	12.09	81.94	1,000
Promod 192	12.35	500.38±22.46	16.19	6.31	77.50	1,298
Summizyme MP	42.65	286.33±11.21	12.16	14.53	73.31	1,493
Flavourzyme	21.76	447.68±13.31	17.24	14.87	67.89	1,966
Neutrase	35.59	236.67±0.26	17.50	21.55	60.95	2,432
Alcalase	11.47	878.35±0.00	52.08	16.61	31.31	6,886
Protamax	24.12	283.73±6.06	38.10	16.79	45.11	4,303

Hydrolysis was performed at 47°C for 24 h. Enzyme: substrate ratio=1:100 (w/w).
[1] Amounts of inhibitors needed for ACE activity inhibition by 50%.
[2] Values present the area within a defined molecular mass distribution, expressed as percent for total area of chromatogram at 220 nm.

Table 2. Recovery yields and IC_{50} values obtained egg albumen hydrolysate with enzyme combinations

Enzyme combination	Hydrolysis time (h)	Recovery yields (%)	IC_{50} value (µg/ml)
Neutrase	24	36.00	283.73±6.06[a]
Neutrase → Flavourzyme	20+4	19.17	300.30±6.47[ab]
Neutrase → Promod 192	20+4	22.50	314.72±1.96[b]
Neutrase → Summizyme MP	20+4	31.76	263.12±6.81[a]
Neutrase → Summizyme LP	20+4	25.0	276.99±0.14[a]

[a, b] Within same rows, means with different superscripts are significantly different (p<0.05).

50% acetonitrile containing 0.1% (v/v) trifluoroacetic acid (TFA, Sigma). The eluate at flow rate of 0.3 ml/min was detected with UV detector at 220 nm. The hydrolysate was diluted in 0.2% mobile phase that was protein-filtered through 0.45 µm syringe filter (Whatman, England), and 50 µl of hydrolysate was applied to the column. The standards, aprotinin (6,500 Da), insulin B fragment (3,495 Da), bradykinin (1,060 Da), GSH (307 Da), and Try (181 Da), were purchased from Sigma, USA.

Measurement of systolic blood pressure

Spontaneously hypertensive male rats (SHR), 14-17 week old and 210-230 g in body weight, were obtained from Samtako Inc. (Korea). They were housed in transparent plastic cages with stainless wire lids in the animal facility of National Livestock Research Center in Korea. SHR was fed standard laboratory diet and had free access to water. Then SHR was randomly assigned to 5 rats per each group and systolic blood pressure (SBP) of SHR was measured as follows. The hydrolysate was dissolved in distilled water and then emulsified with egg yolk by using ultrasonic cleaner for 5 min. Hydrolysate or emulsified hydrolysate was orally administered in SHR by metal zonde in a volume of 0.5 ml. After SHR was kept at 45°C for 10-15 minutes, SBP was measured by tail-cuff method with Power Lab 800 (AD Instruments PtyLtd, Australia).

Statistical analysis

All results were expressed as the mean±standard errors. In the IC_{50} value, significance between the groups was determined by Duncan's multiple range after ANOVA. The statistical significance for antihypertensive test was determined by Student's t-test.

RESULTS AND DISCUSSION

For the production of ACE inhibitory hydrolysates, egg albumen was hydrolyzed with several proteases. Table 1 showed the IC_{50} value, molecular mass distribution and recovery yields. ACE inhibitory activity of neutrase hydrolysate was the highest with IC_{50} value of 236.67 µg/ml. IC_{50}; value of protamax and summizyme MP hydrolysate was 283.73 µg/ml and 286.33 µg/ml, respectively. A hydrolysate digested by alcalase presented the lowest ACE inhibitory activity with 878.35 µg/ml. From the results, the ACE inhibitory activities of egg albumen hydrolysates were dependent on the kind of protease. Enzymatic hydrolysis plays an important role in releasing ACE inhibitory peptides from inactive form within the sequence of proteins (Mullally et al., 1997). The diverse ACE inhibitory activity of the hydrolysates was probably due to the presence of different peptide sequence (Yeum et al., 1992). Neutrase hydrolysate showed the highest ACE inhibitory activity, and was mainly consisted of small peptides less than 1,000 Da in molecular weight. Our result was similar to Hyun and Shin's (2000) report. Recovery yield of neutrase hydrolysate was 35.59%, which was the higher than that of any other hydrolysates.

Table 2 presented the effects of enzyme combinations on the production of ACE inhibitory hydrolysates. The data indicated the little difference between hydrolysates, and decreased by neutrase treatment alone. In addition, recovery yield of hydrolysates with all enzyme combination was rather lower than that of neutrase treatment alone.

Table 3. Effects of enzyme concentrations on recovery yields and IC_{50} values of hydrolysates

Enzyme concentrations (%)	Recovery yields (%)	IC_{50} value (μg/ml)
0.001	5.43	3,118.99±482.55[a]
0.1	10.52	1,117.22±73.10[b]
0.3	13.33	401.89±39.78[c]
0.5	17.92	331.28±3.07[c]
0.7	22.08	315.88±7.79[c]
1.0	35.82	258.85±1.46[d]
3.0	42.75	259.17±1.57[d]
5.0	45.75	257.55±1.20[d]

[a-d] Within same rows, means with different superscripts are significantly different (p<0.05).

Table 4. Effects of hydrolysis on recovery yields and IC_{50} values of hydrolysates in time dependent manner

| Hydrolysis time (h)	Recovery yields (%)	IC_{50} value (μg/ml)
2	13.65	629.55±35.81[a]
4	33.94	256.35±12.83[b]
8	35.25	261.29±1.18[b]
12	33.25	260.29±7.34[b]
24	35.75	269.60±7.63[b]
48	34.75	265.38±0.87[b]

[a-d] Within same rows, means with different superscripts are significantly different (p<0.05).

Decreased ACE inhibitory activity in enzyme combination was probably due to the presence of carboxyl peptidase that may cause the further degradation of ACE inhibitory peptides (Pihlanto-Leppälä et al., 2000).

Table 3 indicated the effect of enzyme concentration on ACE inhibitory activity and recovery yield. Up to 5% enzyme addition, ACE inhibitory activity and recovery yield were increased. With 1.0% enzyme addition, IC_{50} value of hydrolysate was 258.85 μg/ml, but there was no significant difference (p<0.05) up to 5%. The recovery yield was, however, increased by the additional amounts of enzyme. Table 4 indicated ACE inhibitory activity of the hydrolysates according to incubation time. The ACE inhibitory activity was the highest at 4 h incubation. The result implied that the highest activity of egg albumen hydrolysate could be obtained in the early stage hydrolysis, and more than 4 h incubation could not be useful for increasing ACE inhibitory activity. Matsui et al. (1993) also found that with an addition of 1 and 2% of alcalase (*Bacillus licheniformis*) for the hydrolysis of sardine muscle, the maximum activity was revealed within 1 h incubation in proteolysis and then leveled off.

It was concluded from all the above results that neutrase (from *Bacillus subtilus*) was the most adequate enzyme for high productivity of ACE inhibitory hydrolysate, and that the best hydrolysis was fulfilled in 1% enzyme addition and 4 h incubation at 47°C.

There are several reports on the antihypertensive effects
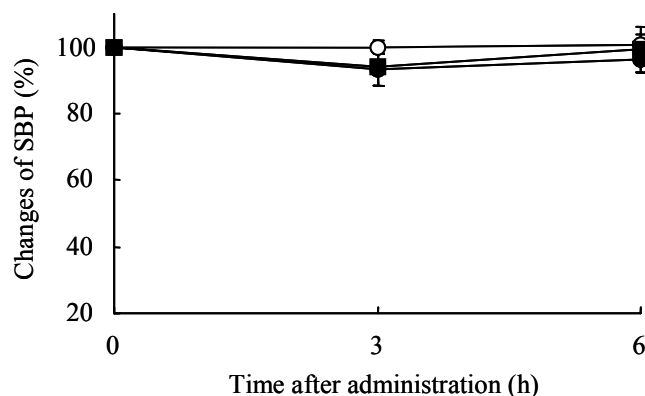
Figure 1. Effects of oral administration of egg albumen hydrolysates on spontaneously hypertensive rats. The change in relative blood pressure at each time was calculated as follows: (Blood pressure-Blood pressure at 0 h)/(Blood pressure at 0 h)×100. Treatments were control (o), 150 mg/kg (■), and 300 mg/kg of body weigh (•) and significant difference between groups was revealed in comparison to the control (* p<0.05, ** p<0.01; t-test, n=5).
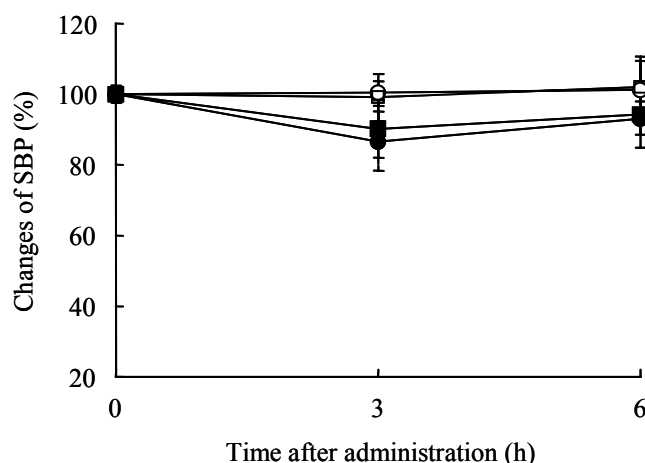
Figure 2. Effects of oral administration of egg albumen hydrolysate emulsified with egg yolk on spontaneously hypertensive rats. The change in relative blood pressure at each time was calculated as follows: (Blood pressure-Blood pressure at 0 h)/(Blood pressure at 0 h)×100. Treatments were control (o), egg yolk (□), 150 mg/kg (■), and 300 mg/kg of body weight (•) and significant difference between groups was revealed in comparison to the control (* p<0.05, ** p<0.01, n=5).

of food protein in relation to the functional peptides in rat and human feeding studies (Suestna and Osajima, 1989; Sekiya et al., 1992; Yamamoto et al., 1994; Nakamura et al., 1996; Hata, et al., 1996; Eto et al., 1999). In SHR experiment, SBP were measured at 0, 3, 6 h after 150 mg/kg and 300 mg/kg oral administration. The average SBP was 206.31±11.46 mmHg before oral administration. Figure 1 showed the change of SBP after administration of the neutrase hydrolysate that was decreased by 6.88% (-14.14

mmHg, p<0.05), observed in 3 h after 300 mg/kg oral administration. However the decreased SBP went back up to the initial level after 24 h (data not shown). These results suggest that ACE inhibitory peptide has a temporary antihypertensive effect by single oral administration (Nakamura et al., 1995).

Although peptides and proteins are more important for drug substance, most peptides and proteins are not stable and, therefore, not effective by oral given (Hilderbrand and Tack, 2000). To avoid destruction of peptide and protein by intestinal proteases, emulsification and microencapsulation techniques have been frequently used (Shively, 1997). Figure 2 showed an antihypertensive effect of oral administration of emulsified egg albumen hydrolysate. SBP was decreased by 13.33% (-27.72 mmHg, p<0.05) after 3 h, at a dose of 300 mg/kg hydrolysate. The decrease was larger than that of administration dissolved in distilled water without emulsification. The egg yolk itself had no effect on the antihypertention. The data have supported that the higher antihypertensive effect of ovokinin was potentiated by emulsion, and phophatidylcholine in egg yolk played an important role in the potent antihypertensive activity by helping the protection from peptidase and the improved intestinal absorption of ACE inhibitory peptides (Fujita et al., 1995).

CONCLUSION

For the production of ACE inhibitory hydrolysates, egg albumen was hydrolyzed with several proteases. The most active hydrolysate was observed by neutrase hydrolysis of egg albumen. IC_{50} value of neutrase hydrolysate was 236.7 μg/ml and the best hydrolysis was fulfilled in 1.0% enzyme addition and 4 h incubation at 47°C. In measuring the antihypertensive effect of emulsified egg albumen hydrolysate, SBP was decreased by 3.33% (-27.72 mmHg, p<0.05) after 3 h, at a dose of 300 mg/kg. The decrement was larger than that of administration dissolved in distilled water without emulsification. These results showed that it is very effective to utilize egg albumen as a protein source for the production of ACE inhibitory peptides. However, further studies are required to investigate the methods to increase recovery yield and the isolation of active peptide is necessary for determining its sequence responsible for ACE inhibitory activity. Economical analysis is required for the industrial usage of egg albumen to produce ACE inhibitory hydrolysate as well.

REFERENCES

Cushman, D. W. and H. S. Cheung. 1971. Spectrophotometric assay and properties of the angiotensin-converting enzyme of rabbit lung. Biochem. Pharmacol. 20:1637-1648.

Eto, Y., T. Ito and S. Nishioka. 1999. Antihypertensive effect of alkaline protease hydrolysate of whey protein on blood pressure in spontaneously hypertensive rats. J. Jpn. Soc. Nutr. Food Sci. 52:301-306.

Feria, S. H. 1965. A bradykinin potentiating factor (BPF) present in venome of Borthtops jararaca. Br. J. Pharmacol. 24:163-165.

Fujita, H., R. Sasaki and M. Yoshikawa. 1995. Potentiation of the antihypertensive activity of orally administered ovokinin, a vasorelaxing peptide derived from ovalbumin, by emulsification in egg phosphatidylcholine. Biosci. Biotech. Biochem. 59:2344-2345.

Hata, Y., M. Yamamoto, M. Ohni, K. Nakajima and Y. Nakamura. 1996. A placebo-controlled study of the effect of sour milk on blood pressure in hypertensive subjects. Amer. J. Clin. Nutr. 64:767-771.

Hildebrand, G. E. and J. W. Tack. 2000. Microencapsulation of peptide and protein. Int. J. Pharmaceuticus. 196:173-176.

Hyun, C. K. and H. K. Shin. 2000. Utilization of bovine plasma protein for the production of angiotensin-I converting enzyme inhibitory peptides. Process Biochem. 36:65-71.

Kinoshita, E., J. Yamakoshi and M. Kiluchi. 1993. Purification and identification of angiotensin-I converting enzyme inhibitor from soy sauce. Biosci. Biotech. Biochem. 57:1107-1110.

Maruyama, S. and H. Suzuki. 1982. A peptide inhibitor of angiotensin converting enzyme in the tryptic hydrolysate of casein. Agric. Bio. Chem. 46:1393-1394.

Matsui, T., H. Matsufuji, E. Seki, K. Osajima, M. Nakashima and Y. Osajima. 1993. Inhibition of angiotensin I converting enzyme by Bacillus lichenifirmis alkaline protease hydrolysates derived from sardine muscle. Biosci. Biotech. Biochem. 57:922-925.

Miyoshi, S., H. Ishikawa, T. Kaneko, F. Fukui, H. Tanaka and S. Maruyama. 1991. Structures and activity of angiotensin-converting enzyme inhibitors in an α-zein hydrolysate. Agric. Bio. Chem. 55:1313-1318.

Mullally, M. M., H. Meisel and R. J. FitzGerald. 1997. Angiotensin-I-converting enzyme inhibitory activities of gastric and pancreatic proteinase digests of whey proteins. Int. Dairy J. 7:299-303.

Nakamura, Y., N. Yamamoto, K. Sakai and T. Takano. 1995. Antihypertensive effect of sour milk and peptide isolated from it that are inhibitors to angiotensin I-converting enzyme. J. Dairy Sci. 78:1253-1257.

Nakamura, Y., O. Masuda and T. Takamo. 1996. Decrease of tissue angiotensin I-converting enzyme activity upon feeding sour milk in spontaneously hypertensive rats. Biosci. Biotech. Biochem. 60:488-489.

Peart, W. S. 1976. The Renin-angiotensin System: Peptide Hormones. The Macmillan Press Ltd.

Pihlanto-Leppälä, A., P. Koskinen, K. Piilola, T. Tupasela and H. Korhonen. 2000. Angiotensin I-converting enzyme inhibitory properties of whey protein digests: concentration and characterization of active peptides. J. Dairy Res. 67:53-64.

Sekiya, S., Y. Kobayashi, E. Kita, Y. Imamura and S. Toyama. 1992. Antihypertensive effects of tryptic hydrolysate of casein on normotensive and hypertensive volunteers. J. Jpn. Soc. Nutr. Food Sci. 45:513-517.

Shively, M. L. 1997. Protein Delivery Physical System: Multiple

Emulsions for the Delivery of Protein. Plenum Press.

Suetsuna, K. and K. Osajima. 1989. Blood pressure reduction and vasodilatory effects *in vivo* of peptides originating from sardine muscle. J. Jpn. Soc. Nut. Food Sci. 42:47-54.

Yamamoto, N., A. Akino and T. Takano. 1994. Antihypertensive effect of the peptide derived from casein by on extracellular proteinase from *Lactobacillus heveticus* CP790. J. Dairy Sci. 77:917-922.

Yeum, D. M., T. G. Lee and H. S. Byun. 1992. Angiotensin I converting enzyme inhibitory activity of enzymatic hydrolysates of mackerel muscle protein. Bulletin Korean Fish Society 25:229-235.