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Protein Hydrolysate Batch Production with Angiotensin I-Converting Enzyme Inhibitory Activity from Egg Whites

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ABSTRACT

Peptides derived from egg whites by thermolysin digestion were fractionated and characterized to investigate their inhibitory activity against angiotensin I-converting enzyme (ACE). The antihypertensive effect of egg white hydrolysates in strain SHR spontaneously hypertensive rats was also investigated. At optimal conditions, pH 8, 60°C, and (E)/(S) = 0.02%, digestion of 1% crude egg white solution by thermolysin was carried for 4 hours. The recovery yield from the supernatant after centrifugation was around 80%. The hydrolysate showed high ACE inhibitory activity (IC_{50} = 33 μg/mL) and imparted neither bitter taste nor the iron odor of egg whites. Sequential ultra-filtration of hydrolysate with MW cut-off 10,000, 3,000 and 1,000 Da resulted in increased activity from each filtrate up to IC_{50} = 17 μg /mL. Thermolysin was recycled from the hydrolysate for subsequent batch use for a total of four batches without reduction in anti-hypertensive activity. The hydrolysate demonstrated an anti-hypertensive activity in spontaneously hypertensive rats at an orally administrated dosage of 0.2 g/kg body weight.

Key words: angiotensin I-converting enzyme (ACE), antihypertensive activity, protein hydrolysate, egg whites.

INTRODUCTION

Peptides derived from food protein hydrolysates as potential nutraceuticals have been extensively studied for many years. Among the peptides with anti-hypertensive activity, angiotensin I converting enzymes (ACE) inhibitors have been obtained from the proteolytic products of many food proteins[1-2]. Studies on ACE inhibitory peptides have been focused mainly on the activities of purified peptides and their structure. Ovokinin, a pentapeptide with anti-hypertensive effect, has been isolated and characterized from ovalbumin tryptic hydrolysate[3-4]. Potent derivatives have been designed from ovokinin[5-6]. Gene encoded ovokinin (2-7) has been incorporated in soybeans to create modified soybean protein with anti-hypertensive activity[7]. Regarding the application of anti-hypertensive substances as functional foods, the production of active peptide fractions is more important than the active peptide purification. A few antihypertensive functional foods from casein hydrolysates, soy isolate and banito have been developed in Japan[8]. In the production of active peptides, high peptide productivity, protein source and proteolytic enzyme availability must be fulfilled. High productivity can be achieved by producing more active peptide fractions at higher yield. The cost for the protein source and proteolytic enzyme should be kept sufficiently low for industrial use.

In 2004, 23.5 million laying birds in Taiwan produced 256 eggs each. The average annual egg consumption per person in this country is up to 275 eggs[9]. The egg industry is important to farmers and eggs are an important animal protein source for people. Eggs contain a variety of proteins that own unique functional and nutritional properties. The egg white is a superior foaming agent in baking, whereas the egg yolk is an excellent emulsifier for mayonnaise[10]. To make its application simple in various food processing, the egg white and egg yolk are usually separated and packaged in the egg processing industry. The egg contains all of the essential amino acids and is commonly used as a standard for protein quality assessment[11]. Because of its balanced amino acids, the egg has been investigated to produce hydrolysate as nutraceuticals with or without anti-hypertensive activity[12-13]. This study further evaluates batch production of nutraceutical hydrolysates with anti-hypertensive activity using thermolysin as proteolytic enzyme.

MATERIALS AND METHODS

I. *Enzymes and Chemicals*

Thermolysin (from *Bacillus thermoproteolyticus rokko*) and ACE obtained from rabbit lungs, hippury-L-histidyl-L-leucine (HHL), hippuric acid (HA), molecular weight markers including cytochrome C, aprotinin, gastrin I, substance P, triglycine and glycine were purchased from Sigma Chemical Co. (St. Louis, MO, USA) or Merck (Damdstadt, Germany). Regenerated cellulose ultra-filtration membranes were obtained from Millipore Corporation (Bedford, MA, USA).

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II. *Preparation of Hydrolysates*

1.0% (w/v) crude egg white and 0.2% (w/w of egg white) thermolysin was employed to produce hydrolysate. The temperature and pH for thermolysin was determined in our previous study to be 65°C and 8.0, respectively. The pH of egg white solution was adjusted to be 8.0 with 1 N NaOH and heated to 65°C before thermolysin addition. Sample aliquots, withdrawn at 10, 20, 30, 60, 120, 180 and 240 min from proteolytic mixture, were immediately heated in a boiling water bath for 10 min. Parts of heated mixtures were used for measurement of their pH and degree of hydrolysis (DH). The rest of heated mixture was subjected to centrifugation at 10,000 ×g in a microcentrifuge for 10 min. The supernatants were assayed for their ACE inhibitory activity. The DH at any time was measured by orthophthaldialdehyde (OPA) method[14].

III. *Assay for ACE Inhibitory Activity*

ACE inhibitory activity was analyzed spectrophotometrically using HHL as the substrate according to the method of Cushman and Cheung[15]. HHL was prepared with 0.1 M sodium borate buffer (pH 8.3) containing 0.4 M NaCl. ACE from rabbit lung was dissolved in the same buffer at a concentration of 60 mU/mL. A mixture containing 225 µL of HHL solution and 25 µL of protein hydrolysate was incubated at 37°C for 5 min. 75 µL of ACE solution was then added in and the incubation was extended for 30 min. The reaction was stopped with 20 µL of 0.1% trifluoroacetic acid (TFA). HA liberated by ACE was determined by RP-HPLC on a LiChrospher C18 column (4 × 250 mm, Merck, Germany). The mobile phase was 0.1% TFA in 50% methanol with a flow-rate of 0.8 mL/min. The effluent was monitored with an ultraviolet detector (Shimadzu, Tokyo, Japan) at 228 nm. The IC_{50} value was defined as the concentration of ACE inhibitor or protein hydrolysate needed to reduce the height of the HA peak to 50%, and determined by regression analysis of ACE inhibitory activity (%) versus protein concentration. The IC_{50} value was expressed as mg protein/mL. ACE inhibitory activity (%) was expressed as

$$\text{ACE Inhibitory Activity (\%)} = (Ho - Hp) \times 100 / Ho$$

Where H_o is the height of HA peak without protein hydrolysate; H_p is the height of HA peak with protein hydrolysate.

IV. *Effect of Differential Ultrafiltration Membranes on ACE Inhibitory Activity*

Hydrolysates generated by enzymatic hydrolysis were subjected to sequential ultra-filtration using regenerated cellulose discs with 10, 3 and 1 kDa molecular weight cut-offs (MWCO), individually. Each permeate was collected for the determination of protein concentration, profile of molecular weight distribution and IC_{50} for ACE inhibitory activity.

V. *Molecular Weight Distribution*

The molecular weight distribution of the hydrolysate was analyzed by high-performance size-exclusion chromatography (HPSEC). The HPSEC consisted of a Superdex peptide 10/300 GL column, connected to a UV detector set at 214 nm. The mobile phase was 0.02 M phosphate buffer (pH 7.2) containing 0.25 M NaCl and the flow rate was set at 0.5 mL/min.

VI.*Recycling Thermolysin for Batch Process*

The batch reactor is a reaction vessel (250 mL) with a hot water jacket on stir to provide uniform heating and mixing. One hundred milliliters of 1% egg white (pH 8.0) and thermolysin was introduced into the reaction vessel and the mixture was heated. After egg white digestion at optimal condition for 4 hr, the hydrolysate was centrifuged (3,000 ×g) and the supernatant was subjected to ultra-filtration through a membrane with a 3 kDa MWCO. The permeate (about 80% in volume) was recovered for the measurement of ACE inhibitory activity and total nitrogen. The retentate (20% in volume) above the membrane, which contains the thermolysin, was recycled for subsequent batch hydrolysis. The recycling of thermolysin for batch hydrolysis was repeated for three times

VII. *Preparation of Hand-Shaken Multi-Lamellar Vesicles (MLVs)[16]*

Three mL of chloroform solution containing 300 µmole lipid (lecithin:cholesterol = 1:0.25) was rotor-evaporated to form a thin film in round flask under vacuum. Two mL of phosphate buffered saline containing supernatant hydrolysate without further fractionation was added into flask. After addition of few glass beads (1.4 cm in diameter), the whole solution was shaken at 50°C in water bath for 5 min to make the multi-lamellar vesicles. After cooling at room temperature for 10 min, the MLV liposome solution was ready to use.

VIII. *Measurement of Blood Pressure[17]*

Male SHR were purchased from National Laboratory Animal Center (Taipei, Taiwan, ROC). Systolic blood pressure (SBP) of SHR, 7 to 8 wks of age (250 to 300 g body weight), were measured as follows. Rats that had been given each hydrolysate by gastric intubation were kept at 37°C for 5 min, and the SBP were measured by tail cuff with a programmed electro-sphygmomanometer (MK 1030, Muromachi Co. LTD, Japan). Phosphate buffered saline (0.15 M NaCl and 0.01 M phosphate buffer, pH 7.4) containing egg whites was used as a control, and the anti-

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hypertensive effects of the hydrolysate powder dissolved in this buffer were measured.

IX. *Statistical Analysis*

Sensory evaluation for flavor, color and oval acceptance using a 5-point hedonic scale to determine differences among the treatments were carried out by a group of 10 graduate students in the Department of Food Science, Tunghai university. The experimental data were analyzed by variance analyses with significance defined at $p < 0.05$. All statistical analyses were performed with Statistical Analysis System software (SAS Institute Inc., Cary, N.C., USA)

RESULTS AND DISCUSSION

I. *Degree of Hydrolysis and ACE Inhibitory Activity of Hydrolysate*

Thermolysin (EC 3.4.24.27) from *Bacillus thermoproteolyticus rokko*, is a thermostable extra cellular metalloendopeptidase[18] containing four calcium ions. Cofactors are zinc and calcium[19-20]. The thermolysin hydrolyzes peptide bonds on N-terminal sides of hydrophobic amino acid residues[21]. Its optimal pH and temperature for activity were 8.0 and 65-70°C, respectively. Thermolysin is stable from pH 5 to 9.5. Thermolysin has low cleavage specificity, therefore it produces a number of short fragments. The thermolysin preferential cleavage site: X-cleavage-Y-Z, where X = any amino acid, Y = Leu, Phe, Ile, Val, Met, Ala, and Z is any amino acid other than Pro. Four proteases including alcalase, esperase, thermolysin and chymotrypsin have been used to hydrolyze egg white in previous study and thermolysin is the most potent one (data not shown) because of its low specificity. Enzymatic hydrolysis is very costly due to the cost of enzyme. Low dosage of thermolysin was employed in this investigation.

The change in pH, degree of hydrolysis (DH) and ACE inhibition of hydrolyzed mixture during hydrolysis process are shown in Figure 1. The pH dropped quickly from 8 to 6.6 in first 30 min during digestion process and then slowly dropped down to 6.5 in 4 hr.

Production of peptide fragments during digestion process shall increase in acidity. The decrease in protein concentration and the increase in acidity would lower the enzymatic activity, and thus slow down the drop in pH. Activity of thermolysin will remain 60% at pH 6.5. The pH control of hydrolysis mixture by controller during digestion would be an effective way to improve the enzymatic activity. Conversely, the DH during the digestion process increased rapidly from 0 to 11.4% in the initial 30 min. and then slowly increased to 15.1% in 4 hr. Quite similar to the change in DH, ACE inhibitory activity of 10-fold diluted hydrolysate mixture increased rapidly to 57% in first 30 min, 60% in next 30 min and kept almost steady state in following hour digestion. All of these changes in pH, DH and ACE inhibitory activity were of typical enzymatic digestion and of hyperbolic function of the process time. Four hour hydrolysis was considered to be enough for this process.

II. *Characteristics of Hydrolysate*

The clear hydrolysate solution exhibited a very light peptone odor and no bitter taste. The color attributes, L, a and b, for hydrolysate solution and hydrolysate powder were 99.3, -0.9, 5.8 and 93.1, -1.2, 4.93, respectively. The hydrolysate will impart neither color nor flavor to the product when added as a food additive. Most protein hydrolysate with other enzymes, especially bacterial enzyme, will give a bitter flavor. The bitter taste of hydrolysate would limit its use as food additive or consumption as it is. The bland flavor of the egg white hydrolysate digested by thermolysin is due to its special enzymatic specificity, i.e. thermolysin hydrolyzes the peptide bond

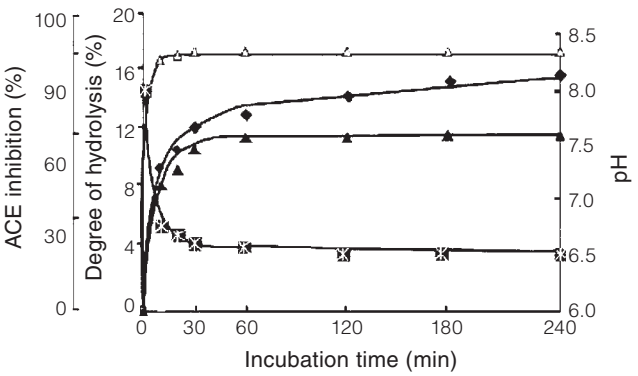

Figure 1. The time course of changes of pH, degree of hydrolysis and ACE inhibitory activity of egg white solution hydrolyzed by thermolysin.
◆ Degree of hydrolysis
△ ACE inhibition of original hydrolysate
▩ pH value
▲ ACE inhibition of 10 × fold diluted hydrolysate

Table 1. Sensory evaluation of skim milk and skim milk containing egg white hydrolysate.

Product	Odor[a]	Bitterness[b]	Overall acceptability[c]
Skim milk	2.00 ± 0.45^{x}	1.44 ± 0.53^{x}	3.70 ± 0.48^{x}
Skim milk containing egg white hydrolysate	2.33 ± 0.82^{x}	1.50 ± 0.53^{x}	3.56 ± 0.53^{x}

Mean values in the same column that are followed by different letters are significantly different. ($p < 0.05$)
[a] A five-point scale (5 = extremely smell, 4 = moderate smell, 3 = slightly smell, 2 = trace of smell, 1 = no smell).
[b] A five-point scale (5 = extremely bitter, 4 = moderate bitter, 3 = slightly bitter, 2 = trace of bitterness, 1 = no bitter).
[c] A five-point scale (5 = like very much, 4 = like slightly, 3 = neither like or dislike, 2 = dislike slightly, 1 = dislike very much).

on the N-terminal site of hydrophobic amino acid residues. The sensory evaluation of skim milk and skim milk containing 1% egg white hydrolyste powder is shown in Table 1. No significant difference could be found in odor, bitterness and overall acceptability between skim milk and skim milk containing hydrolysate.

III. *Fractionation of Hydrolysate and Inhibitory Activity*

It is generally recognized that only small peptide (MW < 1 kDa) will exert ACE inhibitory activity. A simple and effective way to separate the small peptide fraction is to filter the hydrolysate through a membrane disc with suitable MWCO. The peptide yield and ACE inhibitory activity of each permeate obtained by sequential ultra-filtering hydrolysate with 10, 3 and 1 kDa MWCO membranes are shown in Table 2. The ACE inhibitory activities of permeate increased with a decrease in MWCO. IC_{50} of fractions of permeate through 3 and 1 kDa MWCO membrane were 25 and 14 μg/mL, respectively. The molecular weight distribution of hydrolysate and its permeates from different MWCO membranes are shown in Figure 2. The peptide with molecular weight larger than 10 kDa has been completely removed by 10 kDa MWCO membrane. The molecular distribution profile of 3 kDa permeates is quite similar to that of 1 kDa permeate. For safe recycling of thermolysin for subsequent use and saving processing time, membrane with 3 kDa MWCO was chosen to use in batch production of hydrolysate with ACE inhibitory activity.

Tryptic casein hydrolysate with an IC_{50} value of 166 μg protein/mL has been reported to have an anti-hypertensive effect in spontaneously hypertensive rate[22]. The IC_{50} values obtained from ultra filtered tryptic digests of milk protein range from 130-210 μg protein/mL and this indicated the potential of such protein hydrolysates as nutraceuticals in prevention of hypertension[23]. The IC_{50} values for the hydrolysates and permeates reported herein are even lower and the hydrolysates likely mediate an anti-hypertensive effect in spontaneously hypertensive rats.

IV. *Recycling Thermolysin for Batch Process*

Contrary to most biological enzymes, thermolysin is considerably stable at below 70°C. Its molecular weight is around 34.6 kDa. Thermolysin could be easy to recover from hydrolysate by ultra-filtration for reuse. Peptide concentration and ACE inhibitory activity of 10 × fold diluted hydrolysate from batch process by recycled thermolysin are shown in Figure 3. Both clear hydrolysates and permeate from membrane of 3 kDa MWCO have same ACE inhibitory activity and exhibited almost same activity through four batch processes. The result implied that ACE inhibitory activity of hydrolysate is from small peptides with molecular weight less than 3 kDa. That means that ultra-filtering to recycle thermolysin did not reduce the ACE inhibitory activity of perme-

Table 2. The peptide content, yield and IC_{50} value of egg white hydrolysate and its permeates, respectively, filtered by different MWCO membranes.

Fraction	Protein content	Protein recovery	Inhibition 10X dilution	IC_{50}
	mg/100mL	%	%	μg/mL
Supernatant	77.9	100	61	53.2
fMw < 10 kDa	58.6	75.3	68	33.2
fMw < 3 kDa	36.6	46.9	66	20.5
fMw < 1 kDa	29.8	38.2	66	17.2

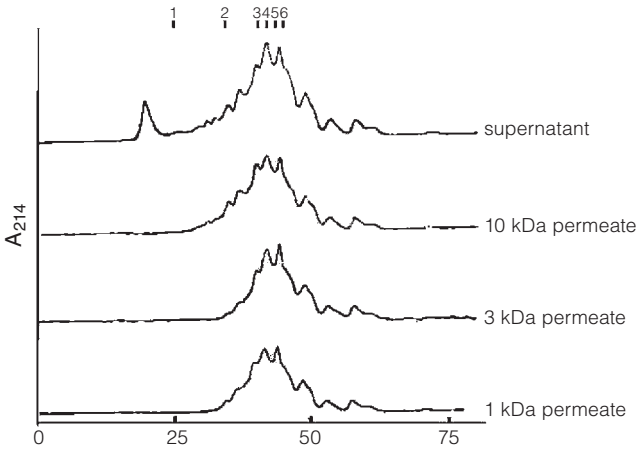

Figure 2. The molecular weight distribution of egg white hydrolysate supernatant and permeates obtained from reactor with different MWCO membranes.
Mr Standard: (1) Cytocrome C, Mr = 12,500; (2) Gastrin, Mr = 2,126; (3) Substonce P, Mr = 1,384; (4) $(Glycine)_6$, Mr = 360; (5) $(Glycine)_3$, Mr = 189; (6) Glycine, Mr = 75.

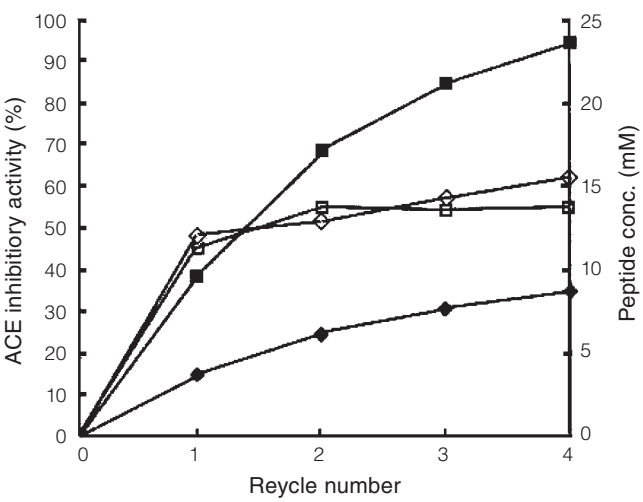

Figure 3. Peptide concentration and ACE inhibitory activity of hydrolysate from batch process by recycled thermolysin
□ ACE inhibitory activity of 10 fold diluted 3 kDa permeate
◇ ACE inhibitory activity of 10 fold diluted supernatant from centrifugation
▩ Peptide concentration of 10 fold diluted supernatant after centrifugation
◆ Peptide concentration of 10 fold diluted 3kDa permeate

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ate. The peptide concentrations in both clear hydrolysate and permeate are increased as the number of thermolysin recycling increases. The peptide concentration in hydrolysate is more than double that of permeate in all batch processes. Increase in peptide concentration in hydrolysate would render ultra-filtering more difficult and more time-consuming. More than four recycling of thermolysin is likely feasible for batch process and the cost of the hydrolysis will be greatly reduced.

V. *Changes of SBP of SHR after Administration of the Hydrolysate*

At 0, 2, 4, 6 and 8 hr after oral administration of the hydrolysates or liposome encapsulated hydrolysate, SBP were measured in SHR rats (Figure 4). In the control, no changes in SBP occurred within 8 hr after administration. However, the peptides by thermolysin showed potent anti-hypertensive activity in SHR rats (186 mg of peptide/kg of body weight) 2 hr after administration (-26.7 mmHg). At 4, 6 and 8 hr after administration, these peptides also exerted potent anti-hypertensive activity (-23, -20 and -13 mmHg, respectively). No significant difference is found between two dosages: 186 mg and 372 mg of peptide/kg of body weight. MLVs-peptide showed better effect on the anti-hypertensive activity than peptide only. Liposome-encapsulation has been employed to deliver drugs to the target because lipsome is resistant to peptic digestion and enhances intestinal absorption[24-25]. These peptides had an anti-hypertensive effect that is dependent on the dose and the preparation given to SHR rats.

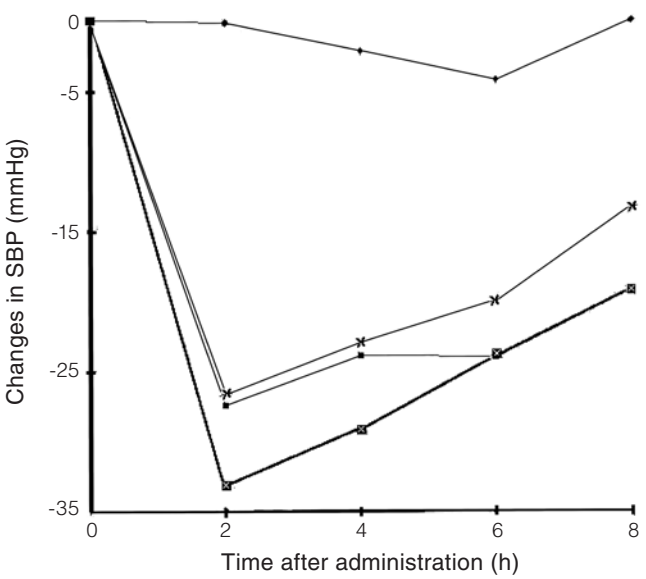

Figure 4. Antihypertensive effect of a single oral administration of hydrolysate in spontaneous hypertensive rats(SHR). Each point is the mean of the changes of systolic blood pressure(SBP) of five SHR.

CONCLUSIONS

Thermolysin showed high efficiency egg white digestion. Egg white hydrolysate using thermolysin is almost white in color and bland in flavor and can be used as a nutraceutical. The peptide mixture in the hydrolysate exhibited strong ACE inhibition activity. The main active peptides in hydrolysate were relatively short with MW below 1kDa. The peptide mixture in the hydrolysate showed a potent anti-hypertensive effect on SBP in SHR rats at 0.18 g/kg of body weight. Recycling thermolysin for peptide mixture batch production was feasible and could be repeated many times to reduce the production cost. These hydrolysate peptide mixtures could be used as a health food additive for hypertensive patients.

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