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Inhibition of Angiotensin I - Converting Enzymes by Enzymatic Hydrolysates from Chicken Blood

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ABSTRACT

This study examined the hydrolysis of chicken blood meal on soluble protein content, peptide content, the degree of hydrolysis and the inhibition of the Angiotensin I - Converting Enzyme (ACE). The results showed that soluble protein, peptide content and the degree of hydrolysis of the hydrolysates increased when either hydrolysis time or enzyme concentration increased. Five-hour long hydrolysis, using 10% Alcalase enzyme produced the highest ACE-inhibition activities. Under these conditions, the Alcalase IC_{50} value at 0.34 mg peptide/mL was significantly lower than that obtained from other combinations of enzyme, concentration and hydrolysis time. Separation of the hydrolysates by ultrafiltration isolated a fraction (F3) of less than 3000 Da molar mass. The F3 fraction performed with an IC_{50} value of only 0.06 mg peptide/mL. Further separation by FPLC using a Superdex peptide 10/300 GL gel column produced the highest inhibitory efficiency ratio (1071%/mg/mL). These results suggest that chicken blood hydrolysates can potentially be developed as functional food products in the future.

Key words: chicken blood, hydrolysates, angiotensin converting enzyme inhibition

INTRODUCTION

Modern dietary habits have led to an increase in the prevalence of cardiovascular diseases. This is evident from the fact that hypertension-related diseases account for half of the ten most common causes of death[1]. The Renin-Angiotensin-Aldosterone system controls the body's blood pressure. The angiotensin I-converting enzyme (ACE) plays an important role in this system, using hydrolysis to convert non-active angiotensin I in blood into its active form, angiotensin II. This, in turn, causes blood vessels to contract and blood pressure to rise. In addition, ACE deactivates bradykinin, a nona-peptide that causes blood vessels to extend, by removing amino acids from its C-terminus, hence also causing blood pressure to rise. Thus, successful repression of ACE activity will lower blood pressure[2,3].

Commonly prescribed medications for hypertension include Captopril, Enalapril, and Lisinopril, all of which are ACE inhibitors (ACEIs). However, they commonly cause side effects such as coughing and dizziness. Physiological and biochemical research in recent years have shown that protein in food is not only a source of amino acids, but can also provide bio-active peptides after digestion or food processing[4]. These peptides can prevent hypertension, oxidation, boost immunity, fight viral infections and lower cholesterol levels. There were reports of peptides with ACE inhibitory properties extracted from food such as beef, pork, chicken, and fish skin[5,6]. Although ACEIs extracted from food are not as potent as their chemically synthesized counterparts, they have relatively few side effects[5,6] and are suitable for daily use as health products.

Chicken blood meal contains 80% of crude proteins, and its amino acid profile is rich in lysine, arginine, methionine and cystine[7]. Most of the chicken blood produced during the slaughtering process is processed into dry food for animals or treated as waste. This is wasteful and is a potential source of environmental pollution. Hence, using the blood as a resource and product will help reduce the waste produced from the slaughtering process. In this study, chicken blood hydrolysates were produced and resolved into different peptide chains after selective enzymatic hydrolysis. Experiments using various enzymes and hydrolysis times yielded products with active ACEIs. By extracting the most active ACEIs from blood, the production of chicken blood hydrolysates with anti-hypertensive properties was optimized. The development of chicken blood as a possible health product or food supplement was discussed.

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MATERIALS AND METHODS

I. *Composition of Chicken Blood Meal*

The crude protein, crude fat, carbohydrate, ash and moisture content of chicken blood meal were analyzed using AOAC official methods[8-11].

II. *Preparation of Chicken Blood Meal Hydrolysates*

Fresh chicken blood was acquired from Chareon Pokphand Enterprise, Nantou, Taiwan. The bloodstock was supplemented with 3.8% sodium citrate (an anti-coagulant), frozen for 48 hours before being processed into chicken blood meal, and stored at -20°C.

Chicken blood meal (2.25 g) was dissolved in phosphate buffered saline (PBS) (25 mL, 10 mM, pH 7.4) and centrifuged at 7520 ×g, 4°C, 20 min to obtain the supernatant. Protein concentrations were assessed and adjusted to 40 mg/mL. Alcalase, Prozyme 6, Protease N or Thermolysin was added at 0.5%, 1%, and 10% (w/w, by protein content). At the reaction temperature of 50°C, the pH of the proteases were as follows: Alcalase, pH 8; Prozyme-6, pH 7; Protease-N, pH 8 and Thermolysin, pH 8. The mixture was allowed to react for 2 or 5 h, then placed in boiling water for 15 min to deactivate the enzymes and terminate the reaction. The hydrolysates were placed in an ice bath to cool. Centrifugation (13000 ×g, 5 min) was performed to obtain the supernatant, which were stored at 4°C.

III. *Hydrolysates Analysis*

(I) *Soluble Protein Content*

The Lowry method was used to determine the protein content of the hydrolysates[12]. This method is based on the reaction of Folin phenol reagent with protein chain tyrosine residues[13]. Diluted samples of hydrolysates (5 μL) were mixed with 25 μL of Reagent A and 200 μL of Reagent B (Bio-Rad Dc Protein Assay Kit) and allowed to settle in the absence of light for 15 min. The optical density at 750 nm was determined using an ELISA reader. The soluble protein content in mg/mL was calculated from a standard curve obtained from bovine serum albumin.

(II) *Peptide Content*

o-Phthaldialdehyde indicators (OPA) react with amines in proteins, peptides and amino acids. Thus, OPA is suitable as a test for the peptide content in hydrolysates[14]. The hydrolysates were filtered through 0.45-μm filters and then diluted. Diluted hydrolysate samples (5 μL) were extracted, mixed with OPA (200 μL), shaken and allowed to settle in the dark for two min. The optical density at 340 nm was compared against a standard curve obtained from Leu-Gly, and the peptide content in mg/mL was determined.

(III) *Degree of Hydrolysis*

The degree of hydrolysis (DH) was estimated by the change in peptide content and expressed as[15]:

DH (%) = [1 - (peptide content at start/peptide content after hydrolysis)] × 100

IV. *Hydrolysate Inhibition of ACE Activity*

(I) *Testing of ACE Inhibition*

Analysis of ACE inhibition of hydrolysates was performed with reference to the methods reported by Cushman and Cheung[16]. Hydrolysates (75 μL) diluted with boric acid (100 mM) were mixed and shaken with ACE enzymes (75 μL) and placed in a water bath at 37°C for 10 min before being combined with HHL (75 μL). The mixture was placed in the water bath again for 30 min. HHL (250 μL) was then added to terminate the reaction. The product, hippuric acid, was extracted using ethyl acetate (750 μL), shaken for 1 min and centrifuged at 3600 ×g, 5 min. 500 μL of the supernatant was filtered using a 0.45-μm filter and evaporated in an open vessel at 80°C. The residue was combined with de-ionized water (1 mL). 200 μL of the resulting solution was transferred to a 96-well UV plate. Optical density was measured at 228 nm to determine the extent of ACE inhibition.

(II) *Testing of ACE IC_{50} Values*

The hydrolysates were diluted using buffer solutions or de-ionized water to test for ACE inhibition. To determine 50% inhibition rates (IC_{50}), data for 40-60% inhibition rates for three levels of peptide content was used to find the most suitable curvilinear regression. The resulting formula was applied to determine the peptide content required to achieve IC_{50}.

(III) *Calculation of ACE IER Values*

If ACE inhibition is lower than 50% and IC_{50} values cannot be found directly, inhibitory efficiency ratio (IER) values may be used instead. The hydrolysates were diluted using buffer solutions or de-ionized water to an appropriate concentration for the determination of protein content and ACE inhibition rate. The rate was divided by the peptide content to obtain the IER. This provided an estimate for ACE activity. IER and ACE are proportional, so a high IER means greater ACE inhibition.

V. *Ultra-Filtration of Hydrolysates*

The group of hydrolysates with the greatest ACE inhibition IC_{50} values was processed using ultra-filtration. The molecular weight cut-off method (MWCO) was used to differentiate the larger molecular weights. The hydrolysates were filtered through two different

membrane filters (MWCOs of 10 KDa and 3 KDa) and centrifuged (5000 ×g, 30 min) to separate into three parts: MWCO of larger than 10 KDa, 3 - 10 KDa and below 3 KDa. The isolated products were then collected for further ACE inhibition testing.

VI. *Fast Performance Liquid Chromatography (FPLC)*

Each molar mass group of hydrolysates was eluted by FPLC using Superdex peptide 10/300 GL tubes (Pharmacia, USA) (MW: 7000-100, size 1.0 × 30 cm). The fractions were individually frozen and dehumidified. Forty milligrams of the meal with the highest ACE IC_{50} values was extracted and buffered using 1 mL PBS and run through 0.22-μm filters to extract 100 μL of liquid sample for analysis. Phosphoric acid (0.05 M) and NaCl (0.15 M) were used as the PBS. A flow of 0.5 mL/min and a pressure of 0.58 MPa were used to differentiate the collected liquid (1 mL per tube). The optical density at 215 nm was analyzed to determine the molecular distribution in each group of hydrolysates. The molecular weight standards used were: Insulin B chain, 3495.9 Da; Gastrin I, 2126 Da; Substance P, 1348 Da and Glycine, 75 Da.

VII. *Testing Fractions for ACE Inhibition*

Optical density measurements produced five peaks representing different molecular masses. Samples of each peak were collected, frozen, dehumidified, and dissolved in buffer solutions and filtered through 0.22-μm filters before testing for ACE inhibition.

VIII. *Statistical Analysis*

Data was analyzed using the Duncan test with the Statistical Package for the Social Sciences (SPSS). A value of $p < 0.05$ was considered to be statistically significant.

RESULTS AND DISCUSSION

I. *Composition of Chicken Blood Meal*

Chicken blood meal contains 65.73% crude protein, 7.71% crude fat, 7.52% carbohydrate, 1.42% moisture and 17.62% ash (Table 1). The crude protein in chicken blood meal is higher than those in chicken bone (23.54%)[15] and breast meat (22%)[17]. Blood from domestic chickens is a suitable source of soluble protein for the health product industry.

Table 1. Composition of chicken blood meal

Item	Crude Protein (g/100 g)	Crude Fat (g/100 g)	Moisture (g/100 g)	Ash (g/100 g)
Blood Meal*	65.73 ± 0.91	7.71 ± 0.03	1.42 ± 0.22	17.62 ± 0.33

* Values represent means ± standard deviation of three replicate analyses (n = 3).

II. *Changes in Conditions for the Hydrolysis of Different Enzymes*

(I) *Effect of Hydrolysis Time on Chicken Blood Meal Hydrolysates*

Alcalase, Prozyme 6 and Protease-N were used in the hydrolysis of chicken blood meal for 2 and 5 h. Soluble protein levels, peptide content, degree of hydrolysis and extent of ACE inhibition (Figure 1A) were tested. For the three proteases, soluble protein concentration increased with the duration of hydrolysis. The use of 10% Alcalase and 10% Prozyme-6 attained the highest levels of soluble proteins, approximately 34 mg/mL, after 5 h of hydrolysis. Hydrolysis of clam protein using Protamex showed similar results[18]. This suggested that prolonged reaction with proteases hydrolysed the otherwise insoluble high molecular weight proteins. Continued hydrolysis cleaved the longer chain, high molecular weight proteins into many smaller peptides. Each cleavage exposed an additional N- and C-terminus and resulted in higher levels of soluble proteins and smaller peptide chains, thus increasing the solubility[19].

As seen in Figure 1B, longer periods of hydrolysis also increased peptide content. Figure 1C demonstrates increased hydrolysis with time for all groups. 10% Prozyme-6 reacting for 5 h achieved the highest peptide concentration (34 mg/mL, 96%). Other enzymes and conditions showed lower performances. Increased hydrolysis also resulted in an increase in amino nitrogen content[20]. The soluble protein content, peptide content and degree of hydrolysis of chicken blood meal hydrolysates were all higher for the three enzymes than the control group. ACE inhibition results in Figure 1D suggest that there is a positive correlation between the time allowed for hydrolysis and the extent of ACE inhibition. ACE inhibition after a ten-to-one dilution showed that hydrolysis with Alcalase for 5 h produced the best results (91%). Other test groups also showed noticeable differences.

(II) *Effect of Protease Concentration on Chicken Blood Meal Hydrolysates*

Experiments to study the effects of varying protease concentration from 1% to 10% on blood meal hydrolysates found that soluble protein levels increased with protease concentration for all molar mass hydrolysate groups, with 10% Alcalase and 10% Prozyme 6 yielding the highest results (Figure 1A). Peptide content and degree of hydrolysis also increased with protease concentration (Figures 1B and 1C). As the hydrolysis reactions proceeded, changes in the enzyme concentration to substrate ratio

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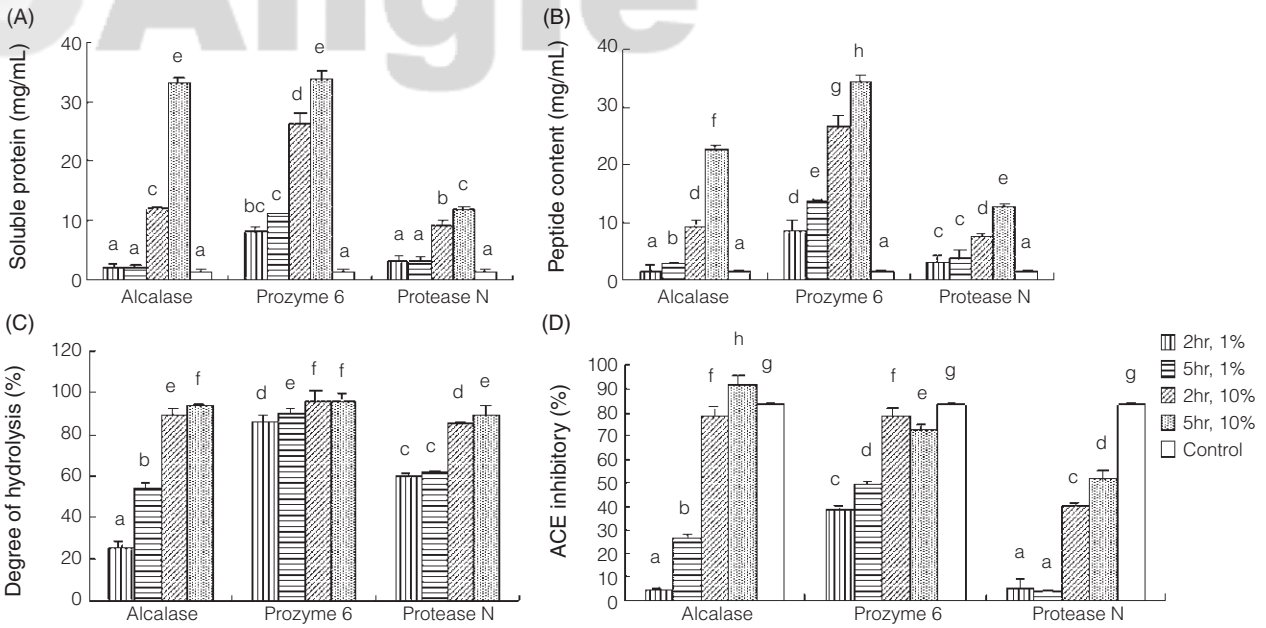

Figure 1. Effects of various chicken blood meal hydrolysates treated with different proteases and times: (A) soluble protein, (B) peptide content, (C) degree of hydrolysis, and (D) ACE inhibition.* Control: The means shown in Figures (A) and (B) were obtained without the addition of enzymes in Figure (D) was obtained using 0.01 μg/mL Captopril. The data in this figure represents the mean ± standard deviation (n = 3). The means (bar value) with different letters are significantly different ($p < 0.05$).

affected the rate of hydrolysis. Thus, it was observed that protease concentration affected the hydrolysis reaction and the higher the concentration of protease, the higher the rate of reaction. Hydrolysis with 10% Alcalase for five hours showed the greatest ACE inhibitory activity of 91% (Figure 1D). Alcalase is an alkaline protease for industrial applications, produced from *Bacillus licheniformis*. The main enzyme component of Alcalase is subtilisin Carlsberg (a serine protease)[21]. This study showed that hydrolysates produced from the hydrolysis of Alcalase achieved the highest inhibition of ACE[20]. Other studies had also used Alcalase in the hydrolysis of bovine plasma proteins, which produced better ACE suppressants than other products of enzymatic hydrolysates[21].

III. *50% Inhibition of ACE (IC_{50}) by Chicken Blood Meal Hydrolysates*

It was of interest to study the peptide concentration required for 50% inhibition of ACE activity, also known as the IC_{50} value. Preliminary measurements of the soluble protein content, peptide content and the degree of hydrolysis showed that chicken blood hydrolysed for 5 h with 10% Alcalase gave the lowest ACE IC_{50} values. The results showed that with a 2% concentration of Alcalase, the ACE IC_{50} was only 1.83 mg peptide/mL (Table 2). In addition, better inhibition results required concentrations greater than 4% and the lowest observed IC_{50} value of 0.34 mg peptide/mL was obtained from the hydrolysis of blood meal with 10% Alcalase (Table 2). Notably, the IC_{50} value of the control group, Captopril (0.01 μg/mL), was 0.0004 mg peptide/mL. At this point in the work, the hydrolysates were not pure. After identifying the most promising candidates, ultra-filtration was applied to obtain the compounds in the pure form.

IV. *ACE IC_{50} Values of Ultra-Filtration Samples*

After selecting the most promising blood hydrolysates, three groups (F1-3) were prepared using centrifugal ration: F1 (> 10,000 Da), F2 (3,000 - 10,000 Da) and F3 (< 3000 Da). The groups were tested for ACE inhibition and F3 showed the lowest IC_{50} values (IC_{50} = 0.06 mg peptide/mL, approximately 0.22 mg/mL) (Table 3). F3 was isolated using a UF membrane with a 3000 Da molecular weight cutoff. Theoretically, this membrane could allow peptides of less than 22 amino acids (assuming that the average MW of amino acids is 137 Da) to pass through and be collected in the permeate[22]. F1 showed the lowest performance with IC_{50} = 0.47 mg peptide/mL. This was due to the presence of large proteins, which had a lower combination frequency with ACE. In fact, F1 showed poorer results than the un-grouped hydrolysates. With the exception of the high IC_{50} values of F1, IC_{50} values decreased in parallel with the group molecular weights. From this, it can be deduced that the most effective inhibitory compounds are among peptides of lower molecular weights. Previous studies presented similar findings: centrifugal filtration of protease hydrolysates in beef increased ACE

Table 2. Changes in ACE inhibitory activity as IC_{50}* values during the hydrolysis of chicken blood meal

Treatment	Time (h)	IC_{50} (mg peptide/mL)
Control**	5	0.004 ± 0.01^{a}***
2.0% Alcalase	5	1.83 ± 0.43^{f}
4.0% Alcalase	5	0.86 ± 0.09^{cde}
6.0% Alcalase	5	0.64 ± 0.31^{bcd}
8.0% Alcalase	5	0.47 ± 0.25^{bc}
10% Alcalase	5	0.34 ± 0.23^{b}
10% Prozyme 6	5	1.16 ± 0.06^{e}
10% Protease N	5	0.98 ± 0.01^{de}

* IC_{50}: The concentration required to inhibit 50% of the ACE activity.
** Control: 0.01μg/mL Captopril.
*** Data represents mean ± standard deviation (n = 3). Values in the same column with different letters are significantly different ($p < 0.05$).

Table 3. The ACE inhibitory activity of chicken blood hydrolysates separated by ultra filtration

Fraction No.	Molecular Weight (Da)	Inhibitory (%)	Peptide Conc. (mg/mL)	IC_{50} (mg peptide/mL)
1	>10000	85.23 ± 0.17	8.41 ± 0.10	0.47 ± 0.006^{a}**
2	10000 - 3000	92.53 ± 0.19	7.71 ± 0.07	0.18 ± 0.001^{b}
3	< 3000	93.29 ± 0.41	5.16 ± 0.02	0.06 ± 0.002^{c}

* IC_{50}: The concentration required to inhibit 50% of the ACE activity.
** Data represents mean ± standard deviation (n = 3). Values in the same column with different letters are significantly different ($p < 0.05$).

Table 4. The ACE inhibitory activity of peptide fractions separated by Superdex peptide 10 / 300 gel chromatography

Peak No.	Molecular Weight (Da)	Peptide Conc. (mg/mL)	IER* (%/mg/mL)
1	2410 - 1570	0.165 ± 0.021	227.45 ± 2.23^{a}**
2	1440 - 1370	0.132 ± 0.032	252.12 ± 0.85^{b}
3	1300 - 1160	0.085 ± 0.007	361.17 ± 0.74^{c}
4	1020 - 880	0.052 ± 0.002	716.41 ± 1.56^{d}
5	470 - 190	0.024 ± 0.001	1071.66 ± 1.03^{e}

* IER (inhibitory efficiency ratio) = % inhibition/ peptide concentration (mg/mL).
** Data represents mean ± standard deviation (n = 3). Values in the same column with different letters are significantly different ($p < 0.05$).

inhibition[23], filtration of protease hydrolysates of chicken breast, which produced fractions with a molecular weight less than 1000 Da, had superior ACE inhibition[24], protease hydrolysate collagen extracts of chicken feet showed better results in solutions containing proteins of less than 3000 Da in molecular weight[25], and the use of different fractions of molecular weight in ultrafiltration in the protease hydrolysis of beef plasma can also increase inhibition[21]. In other studies, however, Alcalase produced ACE inhibitor peptides from bovine blood plasma proteins and the Alcalase hydrolysate of albumin showed high activity (IC_{50} = 0.56 mg/mL)[21]. The hydrolysate produced by the hydrolysis of porcine red blood corpuscles with a mixture of trypsin, chymotrypsin, and thermolysin exhibited angiotensin I-converting enzyme (ACE) inhibitory activity (IC_{50} = 0.58 mg/mL)[22]. In accordance with these reports, the F3 (< 3000 Da) group samples showed the best results with IC_{50} values of 0.22 mg/mL.

V. *ACE IER Values of Chicken Blood Meal Hydrolysates after Ultra-Filtration and FPLC*

The filtrates were frozen after preliminary ultrafiltration. The F3 blood-meal group, with the lowest IC_{50} value, was run through FPLC with a Superdex peptide 10/300 GL column. PBS buffer was used to differentiate the molecular weight distribution in the samples.

The results showed that after filtration with Superdex peptide 10/300 GL, most F3 samples had average molecular weights of less than 3000 Da. The larger the mass of the sample, the shorter its elution time. Conversely, smaller molecule peptides were eluted last. Five peaks (P1-P5) were obtained from the samples as determined by optical density measurements at 215 nm. Table 4 shows the results of the determination of peptide content, ACE inhibition and inhibitory efficiency ratio (IER). The portions with molecular weights between 2500 - 1200 Da showed little variation in IER (200 - 250%/mg/mL). In addition, IER increased when the molecular weight was less than 1000 Da, and P5 (470 - 190 Da) showed the strongest ACE inhibition, with an IER of 1071%/mg/mL. Multiple studies in recent years have shown that most peptides with good ACE inhibition are short peptides of 2 - 10 amino acids[26], and the smaller the molecule after hydrolysis, the greater the effectiveness in lowering blood pressure[21]. Thus, products with good ACE inhibition properties contain low molecular weight peptides. Active small molecule peptides are still unpredictable once they enter the human body. However, studies have shown that compared to larger peptides, smaller peptides are more easily absorbed by the large intestine[27].

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