Production of Antihypertensive Elastin Peptides from Waste Poultry Skin

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***Abstract*—Poultry by-products are not often processed into high-value products. Chicken skin can be considered as one of the most under-utilized by-products and usually been removed for its stigma of high fat content. However, considering that it contains 10-22% protein, poultry skin could be utilized to produce elastin, which is often being incorporated in the production of functional food, cosmetics industry and regenerative medicine. Extracting elastin provides a huge challenge but even bigger opportunities as it has been proven beneficial in the production of functional food. In this study, water-soluble elastin was successfully extracted from broiler and spent hen skin using four different solvents including NaCl, acetone, NaOH and oxalic acid (solubilizing liquid) prior to freeze-drying. The extracted elastin was tested to confirm the purity using amino acid profiling. The water-soluble elastin was analyzed for ACE inhibitory and SDS-PAGE test. It was evident that extracted methods produced high quality elastin comparable to that of commercial elastin, with low amount of Methionine and Histidine. ACE inhibitory activity is present in chicken skin hydrolysate. The ultra-filtrated elastin fraction of broiler and spent hen skin hydrolysate with size ≤3KDa has highest ACE inhibitory activity. The results of the study are the first report on the elastin extracted from broiler and spent hen skin hydrolysis with commercial enzymes could provide the peptides with ACE inhibitory activities. In conclusion, elastin from poultry skin could be a potential new source of natural antioxidant and antihypertensive ingredient for use in food, drinks and cosmetics application.**

***Index Terms*—elastin, extraction, broiler skin, spent hen skin, ACE inhibitory activity**

I. Introduction

Chicken meat is one of the important sources of protein and its consumption has increased dramatically in recent years because there is no religious prohibition against chicken compared to other meats. Broiler meat, the meat of young chicken is soft compared to the spent hen. Spent hen is less productive (18-20 months) and is considered as a by-product due to minimal production of eggs. Annually the meat industry regularly produces large quantities of by-product including blood, bones, offal and skin. This by-product represents a cost for the processing industry as well as being an environmental pollution problem [1], [2]. Recently, different methodologies such as aerobic and anaerobic digestion are utilized for the treatment and disposal of this waste [2]. Elastin is a protein that is present in connective tissue together with collagen in such as aorta, lung, dermis, ligament, skin, tendon, blood vessels and vascular wall which provides elasticity to organs [3]. The functional form of the protein is a highly hydrophobic cross-linked polymer that organizes as sheets or fibers in the extracellular matrix [3]-[5]. Theoretically, this protein should be easy to purify by exposing the tissues to high heat and extreme conditions of pH, resulting remaining residues of elastin due to its unique chemical composition and highly cross-linked nature [2], [3]. Elastin is normally presented in vivo as an insoluble, amorphous, hydrophobic and extensively cross-linked protein [6]. It is widely known that hydrolyzing such elastin with an acid or an alkali or treating it with an enzyme gives a water-soluble elastin. Since water-soluble elastin has the ability to use in the field of regenerative medicine such as artificial blood vessels [3], pharmaceutic [7]-[9] and cosmetic industry [10], [11]. There is nuance information were found on the extraction and characterizing of elastin from poultry skin. Antioxidative activity of the hydrolysates obtained from chicken skin by-products has been reported in our previous study [12]. Therefore, this study was carried out to extract water- soluble elastin from potential tissues of broiler and spent hen's skin and to determine their antihypertensive characteristics for future application.

II. Materials and Method

A. Elastin Extraction

Broiler's and spent hen's skin were suspended in a 10vol of 1M NaCl. After 24hrs extraction with constant stirring in a cold room, the homogenate was centrifuged

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(Eppendorf 5804R, Germany) at 11000rpm for 20min for three times. Consequently, the pellet was washed with distillated water, and was defatted with 30vol acetone for 1 hour for three times. The dry skin was then suspended in 30vol of 0.1N NaOH and heated for 15min in a boiling water-bath with constant shaking. After cooling and centrifugation, the residue was extracted again for 45min in 0.1N NaOH at 100 ℃. The residual of NaOH-insoluble material was washed several times in water and lyophilized prior to further analyses. Subsequently, immersion in 0.25M oxalic acid at 2 to 4 times, relative to the insoluble elastin weight, of a solubilizing out at 100 ℃ for 40 minutes. The residue of insoluble elastin re-submersed for solubilizing step as a water soluble elastin [12].

B. Amino Acid Composition

Elastin samples were hydrolyzed in 6mol/l HCl at 110 ℃ for 16h. The hydrolysate was dissolved in deionized water and filtered. The amino acid composition was obtained using a High Performance Liquid Chromatography (HPLC), equipped with a Waters 410 Scanning Flourescence and AccQ Tag column (3.9×150mm). AccQ Tag Eluent A and AccQ Tag Eluent B or 60% acetonitrile acid was used as the mobile phase (flow rate=1ml/ min).

C. Enzymatic Hydrolysis

Elastin was hydrolysed by Elastase and Alcalase at optimum reaction conditions for each of the enzymes used. Two grams of elastin from broiler and spent hen skin containing 61% and 67% (w/w) of crude protein (N×6.25) respectively, were re-suspended in 40mL of water. Alcalase hydrolysis was conducted at pH 8.5, 60 ℃ and Elastase hydrolysed at pH 8.5, 37 ℃. Hydrolysis was carried out for 2, 4, 8, 12, 16 and 24hrs. At the end of the hydrolysis period, all the hydrolysate solutions were heated at 95 ℃ for 5 min to inactivate the enzyme and centrifuged (Eppendorf 5804R, Germany) at 13000g at 4 ℃ for 15min to separate the soluble hydrolysates from the non-soluble substances. The supernatants were lyophilized to obtain the soluble peptide powders which were stored at -18 ℃.

D. Sodium Dodecyl Sulphate-Polyacrylamide Gel Electrophoresis (SDS-PAGE)

Hydrolysate solution was filtered by 0.2μm membrane and separated into large and small molecular weight fractions by ultrafiltration at 4 ℃ using 10kDa Molecular Weight Cut-off (MWCO) membrane (Vivaflow 200, Sartorius, Germany) followed by 3kDa MWCO (Vivaflow 50) membrane to enrich specific hydrolysate fractions. Both membranes were activated by spinning 100ml of deionized water prior to use. This permeate was defined as small peptides with molecular weight less than 3000Da. The molecular weight distribution of elastin hydrolysates at different DH with concentration of sample 10mg/ml were analysed by using tris-tricine system with 16.5% resolving gel and 10% spacer gel 9 [13].

E. Determination of ACE Inhibitory Activity

ACE inhibitory activity of hydrolysates and their purified fractions was assayed by monitoring the release of Hippuric Acid (HA) from the substrate HHL in RP-HPLC system as described by [14]. ACE was dissolved in 100mM Na-borate buffer (pH 8.3) containing 300mM NaCl at a concentration of 10mU/mL. A volume of 50 μL of elastin hydrolysate samples was hydrolyzed with 50 μL of ACE solution at 37 ℃ for 10 min. The mixture was then incubated with 50 μL borate solution containing 10mM HHL for another 30min. The reaction was stopped by adding 100 μL of 1N HCl and 10 μL of each sample was injected onto a Symmetry Shield C_{18} column (4.6mm×250mm, 5mm; Waters, Milford, MA). The column was eluted with 50% methanol in water (v/v) containing 0.1% Trifluoroacetic Acid (TFA) at a flow rate of 1mL/min and the absorbance was measured at 228nm.

The inhibitory activity was calculated using the following equation:

$$\text{Inhibition activity } \% = (Ec - Es/Ec - Eb) \times 100 \quad (1)$$

where *Ec* was the absorbance of the control sample, *Es* was the absorbance of the hydrolysate sample and *Eb* was the absorbance when the stop solution was added before the reaction occurred (blank). The IC_{50} value was defined as the concentration of peptide in mg/mL required to reduce 50% of the absorbance of the HA peak (50% inhibition of ACE), and determined by regression analysis of ACE inhibition (%) versus peptide concentration.

III. Results and Disscussion

A. Amino Acid Composition

The amino acid compositions of broiler and spent hen skin elastin's and commercial soluble elastin from bovine neck ligament are as summarized in Table I. Glycine was the most dominant amino acid in both source of elastin, by which broiler skin elastin showed a relatively higher content of glycine (20.2%) than that of spent hen's (20.05%). Elastin is known to have a highly distinctive amino acid composition. Starcher [5] reported that elastin in the connective tissue has lack of methionine. Elastin extracted from spent hen and broiler has significantly lower methionine compared to commercial soluble elastin from bovine neck ligament. The amino acid composition result shows that the quality characteristics of the resultant elastin are comparable to that of commercial.

B. Degree of Hydrolysis

Table II shows the degree of hydrolysis of water soluble elastin determined at various incubation times by two selected enzymes: Alcalase and Elastase. DH decreased in early stage of hydrolysis then rapid increase was observed until 12h in both enzymes, after that slightly decreased and remained constant until the end of hydrolysis. The typical hydrolysis results were also reported for fish [15], [16], tuna waste [17], shrimp [18] meat and pig bone wastes [19]. The higher DH indicates

higher activity of Alcalase toward broiler skin proteins at 12h. Generally, alkaline proteases, including Alcalase, exhibit higher activities than do acid or neutral proteases. Therefore, the susceptibility, to hydrolysis, of poultry skin proteins depends on the type of enzyme used.

TABLE I. AMINO ACID COMPOSITION OF ELASTIN FROM BROILER AND SPENT HEN SKIN AS COMPARED TO COMMERCIAL ELASTIN

Amino acids	Spent hen	Broiler	Commercial
Hydroxy proline	10.78 ±0.22^{a}	9.89 ±0.62^{b}	0.53 ±0.07^{c}
Aspartic acid	7.59 ±0.52^{b}	7.72 ±0.09^{b}	9.70 ±0.04^{a}
Serine	2.33 ±0.02^{b}	1.66 ±0.02^{c}	3.95 ±0.03^{a}
Glutamic acid	11.64 ±0.07^{c}	12.39 ±0.38^{b}	14.61 ±0.03^{a}
Glycine	20.05 ±0.40^{a}	20.20 ±0.12^{a}	3.93 ±0.13^{b}
Histidine	1.24 ±0.09^{b}	1.28 ±0.11^{b}	3.05 ±0.01^{a}
Arginine	5.29 ±0.12^{b}	4.03 ±0.05^{c}	5.52 ±0.04^{a}
Threonine	0.87 ±0.03^{b}	0.63 ±0.02^{c}	3.95 ±0.03^{a}
Alanine	8.49 ±0.18^{a}	8.75 ±0.15^{a}	6.35 ±0.06^{b}
Proline	11.40 ±0.05^{a}	11.55±0.09^{a}	5.27 ±0.35^{b}
Tyrosine	1.56 ±0.02^{b}	1.57 ±0.05^{b}	4.28 ±0.12^{a}
Valine	2.96 ±0.03^{c}	3.17 ±0.05^{b}	5.79 ±0.02^{a}
Methionine	1.81 ±0.07^{b}	1.86 ±0.04^{b}	3.43 ±0.05^{a}
Lysine	4.23 ±0.05^{c}	4.53 ±0.03^{b}	8.57 ±0.04^{a}
Isoleucine	2.31 ±0.02^{b}	2.36 ±0.01^{b}	5.43 ±0.05^{a}
Leucine	4.34 ±0.04^{b}	4.62 ±0.04^{c}	8.36 ±0.13^{a}
Phenylalanine	2.80 ±0.03^{b}	3.46 ±0.81^{b}	4.44 ±0.05^{a}
Cysteine	0.13 ±0.04^{b}	0.11 ±0.02^{b}	2.71 ±0.44^{a}
Tryptophan	0.19 ±0.09^{a}	0.20 ±0.05^{a}	0.15 ±0.01^{a}

TABLE II. EFFECT OF ENZYME AND TIME ON DEGREE OF HYDROLYSIS (%) OF BROILER AND SPENT HEN

Time (hrs)	Broiler		Spent hen	
	Elastase	Alcalase	Elastase	Alcalase
2	47.25±0.19^{a}	52.90±0.19^{bc}	51.89±0.35^{a}	47.20±0.46^{a}
4	44.80±0.19^{ab}	47.44±0.33^{a}	49.63±0.35^{a}	49.28±0.35^{ab}
8	53.08±1.63^{c}	51.39±0.56^{b}	57.96±0.76^{b}	54.32±0.46^{c}
12	64.57±0.82^{d}	66.45±1.32^{d}	65.08±0.52^{c}	58.66±0.46^{d}
16	55.72±0.19^{c}	55.72±0.19^{c}	58.48±1.73^{b}	56.57±0.17^{d}
24	48.75±0.68^{b}	53.84±0.50b^{c}	49.98±0.52^{a}	50.15±0.76^{b}

Values are presented as mean ± SE, n = 3.

C. SDS-PAGE

Fig. 1 presents the electrophoretic pattern of elastin before and after proteolysis by elastase. Proteolysis of elastin extracted by non-enzymatic method resulted in degrading proteins and generation of peptides below 14kDa. Elastin was boiled with oxalic acid to solubilise the protein before the proteolysis. After solubilisation of elastin a number of peptides with molecular weight as low as 3kDa were generated. Therefore, the available cutting sites for elastase became less. Elastin hydrolysate was eluted as a broad peak, covering molecular weight range from 180 to 10KDa; about 70% of total elastin-derived peptides are in the range of 65-12KDa [20].

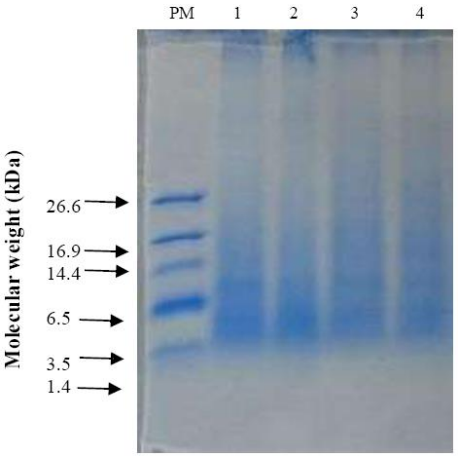

Figure 1. SDS Page of elastin hydrolysates performed using tris-tricine system with 16.5% resolving gel and 10% spacer gel. Each well consists of 24 µg protein. The lane indicated as PM was the protein molecular marker, (1) elastin extracted from Broiler before proteolysis, (2) elastin extracted from broiler after proteolysis, (3) elastin extracted from spent hen before proteolysis, (4) elastin extracted from spent hen after proteolysis.

D. ACE-Inhibitory Activity

Table III shows the ACE-inhibitory activities of extracted elastin and ultra-filtered elastin (molecular weight below 3kDa) from broiler and spent hen skin. The result showed that ACE activity for elastin extracted from broiler is higher than the elastin extracted from spent hen. It was also evident that the ACE activity increased with the reduction of molecular weight. The ultra-filtrated sample fraction of elastin from broiler and spent hen with size ≤3KDa has the highest ACE inhibitory activities of 88.21 and 86.46%, respectively. The naturally occurring peptides with ACE inhibitory activity were first obtained from snake venom. Afterwards, many other ACE inhibitory peptides had been discovered from enzymatic hydrolysis of different food protein. ACE is a dipeptidyl carboxypeptidase that converts an inactive form of decapeptide, angiotensin I, to a potent vasoconstrictor, octapeptide angiotensin II, and inactivates bradykinin, which has a depressor action [21]. Among the bioactive peptides derived from meat proteins, ACE inhibitory peptides have been studied most extensively [22], [23].

TABLE III. ACE-INHIBITORY ACTIVITIES OF EXTRACTED ELASTIN AND ULTRAFILTERED ELASTIN (MOLECULAR WEIGHT BELOW 3KDA) FROM BROILER AND SPENT HEN SKIN

		ACE-inhibitory activity
Extracted Elastin	Broiler	74.18±0.68^{a}
	Spent hen	72.25±0.58^{b}
Ultra-filtered elastin (below 3kDa)	Broiler	88.21±0.25^{a}
	Spent hen	86.46±0.37^{b}

Values are presented as mean ± SE, n = 3.

IV. CONCLUSIONS

The results of the study are the first report on the elastin extracted from broiler and spent hen skin. Chicken

skin is an economically and technologically viable substrate from which to extract elastin. The ACE inhibitory activities were evident in all samples and found to be dependent on the source of elastin (broiler or spent hen skin) and fraction size. Therefore, elastin from poultry skin could be a potential new source of natural antioxidative and antihypertensive ingredient for use in food, drinks and cosmetics application.

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