

**EFFECTS OF ENZYMATIC HYDROLYSIS ON THE ANTIOXIDANT ACTIVITY OF
WATER- SOLUBLE ELASTIN EXTRACTED FROM BROILER AND SPENT HEN SKIN**

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ABSTRACT: Poultry by-products are great economic sources that need to be exploited. Poultry skin could be utilized to extract protein particularly elastin, which is often incorporated in the production of functional food, cosmetic industry or medicine due to its antioxidative properties. In this study, water-soluble elastin was successfully extracted from broiler and spent hen skin and analysed for antioxidant activities including DPPH (1,1-diphenyl-2-picryl hydrazyl), ABTS and metal chelating activity. Antioxidant activity of elastin extracted from broiler skin hydrolysed by Alcalase (EBA) and Elastase (EBE) also elastin extracted from spent hen skin hydrolyzed by Alcalase (ESA) and Elastase (ESE). The EBE, EBA, ESE and ESA had higher DPPH (16-30, 19-35, 29-48 and 31-50%, respectively), ABTS activity (73-79, 60-79, 67-79 and 72-79 %, respectively), and Fe²⁺ chelating activity (65-69, 50-56, 71-77 and 62-70 %, respectively). This concluded that water-soluble elastin is a bioactive component that could potentially be used in the formulation of functional foods, nutraceuticals, cosmetic and pharmaceutical industry.

Key words: Elastin, Extraction, Broiler skin, Spent hen skin, Enzymatic hydrolysis, Antioxidant activity

INTRODUCTION

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 (Arvanitoyannis & Ladas, 2008; Daamen et al., 2007). Recently, different methodologies such as aerobic and anaerobic digestion are utilized for the treatment and disposal of this waste (Arvanitoyannis and Ladas, 2008; Daamen et al., 2007). By- product of chicken such as skin is a great source of proteins and therefore may be viewed as a potential starting material for the production of high value-added products, including elastin. Several studies has been done on characterization and extraction of collagen from poultry (Cliche, Amiot, Avezard, & Gariépy, 2003), fish (Minh Thuy, Okazaki, & Osako, 2014; Muthukumar, Prabu, Ghosh, & Sastry, 2013; Veeruraj, Arumugam, & Balasubramanian, 2013), meat (Lepetit, 2007; Waszkowiak & Dolata, 2007) and DH and antioxidant activity of fish (Intarasirisawat et al., 2012; Klompong et al., 2007; Nasri et al., 2013), meat (Jensen et al., 2014; Onuh et al., 2014; Udenigwe and Howard, 2013) pig by-product (Jensen et al., 2014; Pagán et al., 2013).

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 (Daamen, Hafmans, Veerkamp, & Kuppevelt, 2001). The functional form of the protein is a highly hydrophobic cross-linked polymer that organize as sheets or fibers in the extracellular matrix (Daamen et al., 2001; Getie, Raith, & Neubert, 2003; Starcher & Galione, 1976). 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 (Daamen et al., 2001; Daamen, Veerkamp, van Hest, & van Kuppevelt, 2007). Elastin is normally presented in vivo as an insoluble, amorphous, hydrophobic and extensively cross-linked protein (Duca, Floquet, Alix, Haye, & Debelle, 2004).

It is widely known that hydrolysing 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 (Daamen et al., 2001), pharmaceutical (Dagher, Prior, & Sadeghi, 2010; Hassouneh, MacEwan, & Chilkoti, 2012; Huang et al., 2000) and cosmetic industry (Charlet and Kludas, 1982; Langmaier, Mládek, Kolomaznik, & Sukop, 2002).

Hydrolysis of proteins improves their immunological, bioactive and functional (Intarasirisawat, Benjakul, Visessanguan, & Wu, 2012; Jensen, Dort, & Eilertsen, 2014; Klompong, Benjakul, Kantachote, & Shahidi, 2007; Nasri et al., 2013; Onuh, Girgih, Aluko, & Aliani, 2014; Pagán, Ibarz, Falguera, & Benítez, 2013; Slizyte et al., 2009; Thiansilakul, Benjakul, & Shahidi, 2007a; Udenigwe and Howard, 2013; Zhang, Xiao, Samaraweera, Lee, & Ahn, 2010) properties, making the hydrolysates superior to the native proteins. These properties have been shown to be closely related to the degree of hydrolysis (DH) to which the proteins were hydrolysed. The antioxidant activity of proteins and peptides can be the result of specific scavenging of radicals formed during peroxidation, scavenging of oxygen containing compounds, or metal-chelating ability (Slizyte et al., 2009). There is nuance information were found on the extraction and characterizing of elastin from poultry skin. Therefore, this study was carried out to extract water-soluble elastin from potential tissues of broiler and spent hen's skin and to compare both elastins' characteristics in terms of degree of hydrolysis (DH) and antioxidant activity.

MATERIALS AND METHODS

Chemicals

Alcalase[®] 2.4L (declared activity of 2.4 AU/kg, density of 1.18 g/ml) and Elastase (declared activity of ≥ 4.0 units/mg protein) enzymes, methanol (HPLC grade), sodium chloride, sodium hydroxide, oxalic acid, acetone, hydrochloric acid, sulfuric acid, other chemicals and reagents were analytical grade. All chemical and enzymes were purchased from Sigma-Aldrich (USA).

Elastin extraction

Broiler's and spent hen's skin were suspended in NaCl. After 24 hrs extraction with constant stirring in a cold room, the homogenate was centrifuged (Eppendorf 5804R, Germany) at 11000 rpm for 20 min for three times. Consequently, the pellet was washed with distilled water, and was defatted with acetone for 1 hour for three times. The dry skin was then suspended in 0.1 N NaOH and heated for 15 min in a boiling water-bath with constant shaking. After cooling and centrifugation, the residue was extracted again for 45min in 0.1 N NaOH at 100°C. The residual of NaOH-insoluble material was washed several times in water and lyophilized prior to further analyses. Subsequently, the samples were immersed in 0.25 M oxalic acid for 2 to 4 times, relative to the insoluble elastin weight, of a solubilizing out at 100°C for 40 minutes. The residue of insoluble elastin resubmersed to produce water soluble elastin.

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 ($N \times 6.25$) respectively, were resuspended in water. Alcalase hydrolysis was conducted at pH 8.5, 60°C and Elastase hydrolysed at pH 8.5, 37 °C. Hydrolysis was carried out for 2, 4, 8, 12, 16 and 24 hrs. At the end of the hydrolysis period, all the hydrolysate solutions were heated at 95°C for 5 min to inactivate the enzyme and centrifuged (Eppendorf 5804R, Germany) at 13000 g at 4°C for 15 min 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°C.

Degree of hydrolysis

Degree of hydrolysis was calculated according in percent of trichloroacetic acid (TCA) ratio method as described by Hoyle and Merritt (1994). After hydrolysis, 20 ml of hydrolysis solutions were added to 20 ml of 20% (w/v) TCA to produce 10% TCA soluble material. The mixtures were left to stand for 30 min to allow precipitation, followed by centrifugation (7800 g for 15 min). The supernatant was analyzed for protein content by using Kjeldahl method (AOAC, 2000). The degree of hydrolysis (DH %) was computed as the formula below:

$$DH = \frac{10\% \text{ Soluble nitrogen in the sample}}{\text{Total nitrogen in the sample}} \times 100\%$$

Antioxidant determination

DPPH free radical scavenging activity

The free radical scavenging activity of elastin was determined, using the method of Brand-Williams, Cuvelier and Berset (1995) with a slight modification. Elastin from broiler and spent hen skin with different degree of hydrolysis were dissolved in distilled water to obtain a concentration of (10, 20, 30, 40 and 50 mg/mL). To 100 μ l of sample solutions, 2 ml of 0.2 mM DPPH[•] was added and mixed vigorously. After incubating for 0.5, 5 and 24 hrs, the absorbance of the resulting solutions was measured at 517 nm using a microplate reader (Biotek 259037). The blank was conducted in the same manner, except that distilled water was used instead of sample. DPPH[•] scavenging activity was calculated according to the following equation:

$$\text{DPPH scavenging effect (\%)} = \frac{A_{517 \text{ blank}} - A_{517 \text{ sample}}}{A_{517 \text{ blank}}} \times 100$$

ABTS radical scavenging activity

The ABTS radical scavenging activity method was based on the procedure described by Re et al. (1999). A solution of ABTS^{•+} (7.4 mM) was prepared in 100 mM phosphate buffer saline (PBS), pH 7.4, with 0.15 M sodium chloride, and oxidized using potassium persulfate (2.6 mM) for at 12 hours in the dark. The ABTS^{•+} solution was diluted to a 734 nm absorbance of 0.7 ± 0.05 with ethanol. For measuring antioxidant capacity, 100 μ L of elastin in different concentrations (5, 10, 20, 30, 40 and 50 mg/ml) were added to 2 mL of ABTS^{•+} solution. The absorbance of the above mixture was determined at 734 nm after 10 min using a microplate reader (Biotek 259037).

Ferrous ion chelating activity

Fe²⁺ chelating activity were determined according to the method reported by Decker and Welch (1990) with a few modifications. Briefly, a 2 mL reaction composition containing 200 μ L of elastin (5, 10, 20, 30, 40, 50 mg/mL), 50 μ L FeCl₂ (2 mM), and 1.750 mL deionized water was shaken and placed at room temperature for 5 minutes. A 50 μ L volume of ferrozine (5 mM in methanol) was then added, mixed, left standing for another 5 min, and the 562 nm absorbance of the Fe²⁺ ferrozine complex evaluated against a blank by microplate reader (Biotek 259037). EDTA was used as the positive control and the chelating activity of the extract for Fe²⁺ was calculated as follow:

$$\text{Chelating activity} = \frac{A_0 - A_1}{A_0} \times 100$$

Where A₀ was the absorbance of control (without sample) and A₁ was the absorbance of sample.

Statistical analysis

One-way ANOVA with post hoc Duncan test at significant level 95 % ($p < 0.05$) was carried out using statistical software (SAS). All data were expressed as mean \pm standard error.

RESULTS AND DISCUSSION

Degree of hydrolysis

Figure 1 shows the degree of hydrolysis of water soluble elastin determined at various incubation times by two selected enzymes: Alcalase and Elastase. The enzymatic hydrolysis of both samples proceeded at a rate initially with degree of hydrolysis reaching around 44 to 52% after 4 h incubation, but the hydrolysis rate increased rapidly for elastase and alcalase afterwards. The two enzymes also showed slight differences in the degree of hydrolysis. DH decreased in early stage of hydrolysis then rapid increase was observed until 12 h in both enzymes, after that slightly decreased and remained constant until the end of hydrolysis. The typical hydrolysis results were also reported for fish (Klompong et al., 2007; Slizyte et al., 2009; Thiansilakul et al., 2007a), tuna waste (Guerard, Guimas, & Binet, 2002), shrimp meat (Simpson, Nayeri, Yaylayan, & Ashie, 1998) and pig bone wastes (Pagán et al., 2013). Guerard et al. (2002) confirmed that decline in degree of hydrolysis rate may be due to a reduction in the concentration of peptide bonds present for hydrolysis, enzyme inhibition and enzyme deactivation. The higher DH indicates higher activity of alcalase toward broiler skin proteins at 12 h. Generally, alkaline proteases, including alcalase, exhibit higher activities than do acid or neutral proteases (Klompong et al., 2007). Therefore, the susceptibility, to hydrolysis, of poultry skin proteins depends on the type of enzyme used.

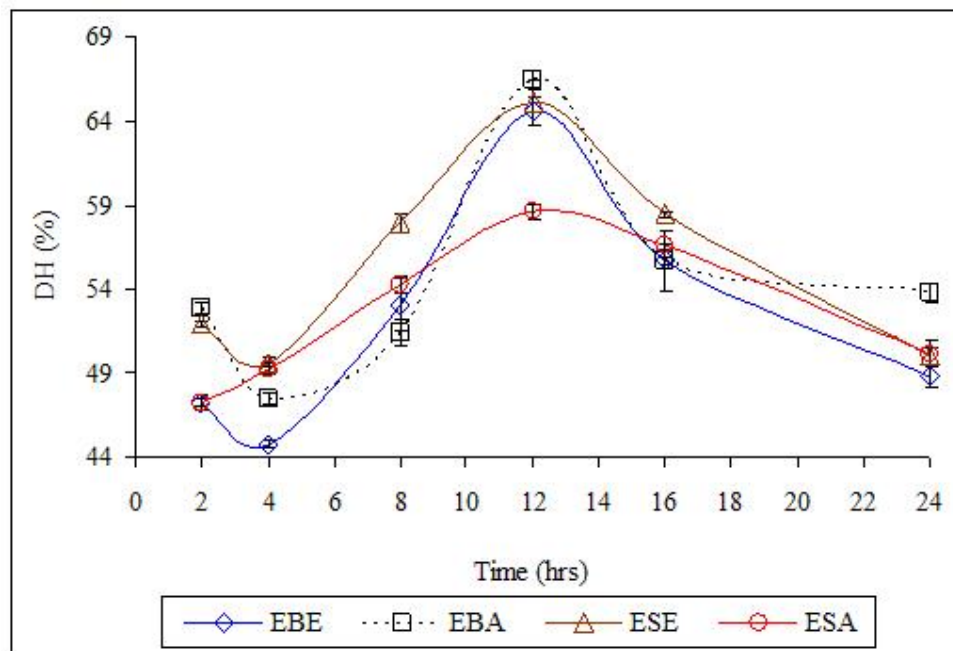


Fig. 1. Progress of hydrolysis of skin protein treated with Elastase and Alcalase.

Note: EBE: Elastin extracted from broiler treated with elastase, EBA: Elastin extracted from broiler treated with alcalase, ESE: Elastin extracted from spent hen treated with elastase, ESA: Elastin extracted from spent hen treated with alcalase.

Table-1: Effect of different enzyme and time of broiler and spent hen skin by ABTS method in 5 (mg/ml) hydrolysis.

Time (hrs)	Broiler		Spent hen	
	Elastase	Alcalase	Elastase	Alcalase
2	79.45±0.79 ^c	64.95±0.12 ^b	67.70±0.48 ^a	73.47±0.30 ^a
4	79.86±0.79 ^c	60.21±0.43 ^a	71.07±0.18 ^b	79.38±0.12 ^c
8	73.20±0.12 ^a	68.87±0.12 ^c	74.23±0.48 ^c	72.58±0.43 ^a
12	77.46±0.18 ^b	73.20±0.93 ^c	77.87±0.56 ^d	77.80±0.50 ^b
16	77.11±0.24 ^b	69.97±0.45 ^c	77.87±1.31 ^d	77.25±0.18 ^b
24	77.04±0.42 ^b	79.45±0.25 ^c	79.18±0.12 ^d	77.11±0.36 ^b

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

Different lowercase in the column (for the same enzyme and different times) indicated significant difference ($p < 0.05$).

Antioxidant properties

The antioxidant activity of the elastin hydrolysates by Elastase and Alcalase at different incubation times was determined by the DPPH free radical scavenging assay, ABTS radical scavenging assay and the ferrous ion chelating activity assay. Based on preliminary data and the different concentrations were selected for each assay. BHT were used as positive controls representing a natural and a synthetic antioxidant, respectively, and EDTA was used as a positive control for ferrous ion chelating ability.

DPPH free radical scavenging activity

Figure 2 shows DPPH free radical activity of elastin extracted from broiler (a) and spent hen (b) skin in different concentrations of elastase and alcalase enzyme. As shown in Figure 2a and b, DPPH value significantly increased with increase of concentration of sample enzymes in both samples, except in broiler sample treated with alcalase after 12 h of hydrolysis was decreased. DPPH radical scavenging assay has been used to estimate the antioxidative activity of compounds as free radical scavengers or hydrogen donors (Klompong et al., 2007).

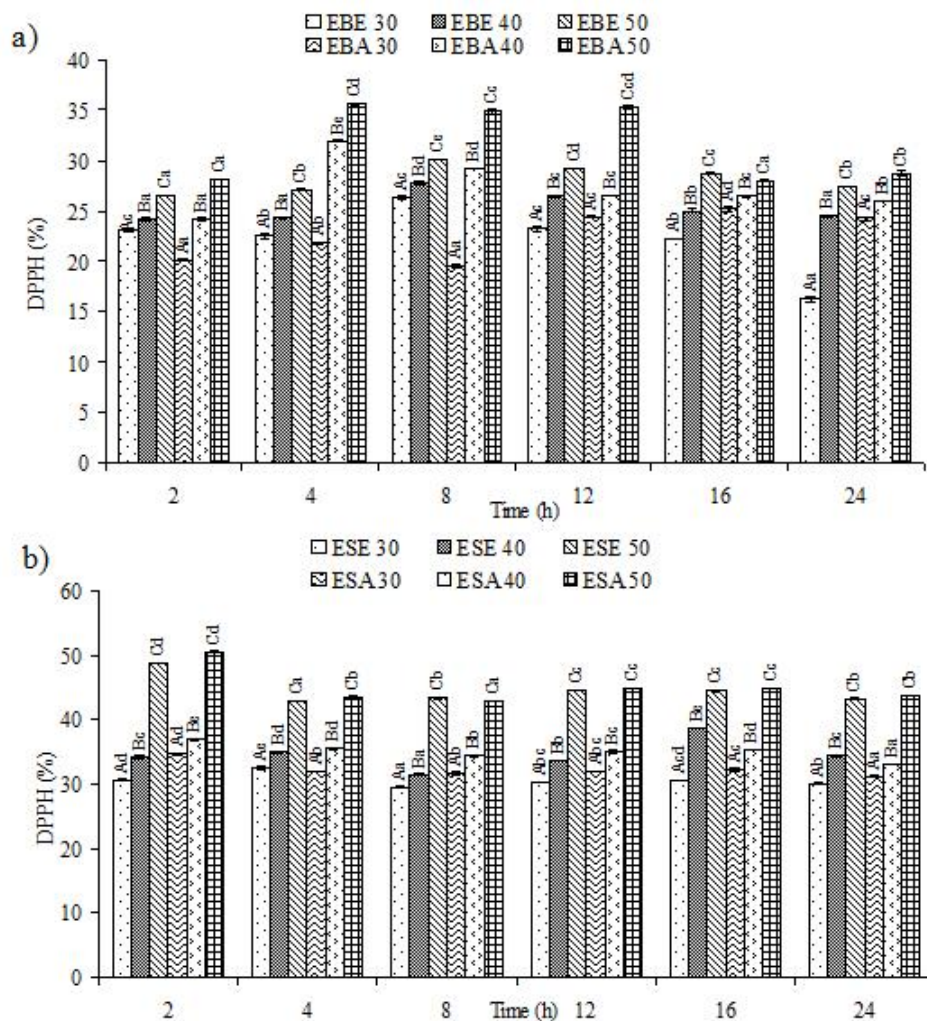


Fig. 2. DPPH radical scavenging activities of broiler (a) and spent hen (b) elastin with different concentrations of enzyme.

Note: Different lowercase (for the same enzyme and different times) indicated significant difference ($p < 0.05$). Different uppercase (for the same enzyme, times and different concentration) indicated significant difference ($p < 0.05$).

EBE: Elastin extracted from broiler treated with elastase, EBA: Elastin extracted from broiler treated with alcalase, ESE: Elastin extracted from spent hen treated with elastase, ESA: Elastin extracted from spent hen treated with alcalase.

DPPH has been stated to be a stable free radical with a maximum absorbance at 517 nm in methanol and as such has been widely used to test reducing substances, especially natural compounds (Girgih, Udenigwe, & Aluko, 2010; Jamdar, Rajalakshmi, & Sharma, 2012). DPPH free radicals are identified when they face a proton-donating substance, such as an antioxidant, leading to a change in colour from purple to yellow as well as a reduction in absorbance. Thus, the DPPH assay is a non-physiologically assay that has been widely used to test the capability of natural compounds to act as free radical-scavengers as a means of evaluating their antioxidant capacities (Kaur and Geetha, 2006). The DPPH scavenging activity was also reported in protein hydrolysate prepared from yellow stripe trevally meat (Klompong et al., 2007) and egg yolk (Sakanaka & Tachibana, 2006) prepared using Alcalase. As the concentration increased from 30 mg/ml to 50 mg/ml the DPPH radical scavenging activity increased. Low DH of ESA in early stage of hydrolysis, the highest DPPH activity was more than 50%. As reported in preliminary study (Nadalian et al., 2013), both samples had high hydrophobic amino acid compounds such as proline and glycine. Increased hydrophobic character of peptides derived from protein sources has been shown to correlate with higher DPPH (Li, Jiang, Zhang, Mu, & Liu, 2008; Pownall, Udenigwe, & Aluko, 2010) when compared with peptide fractions of lower hydrophobic content. However, in the present work, there was no strong relationship between hydrophobic amino acid content and the DPPH scavenging activity.

Zhang, Wang and Xu (2008) also reported that the DPPH radical scavenging activities of rapeseed peptides had no direct correlation with their hydrophobicity. Similar conclusion was also made by Chen, Muramoto, Yamauchi, Fujimoto and Nokihara (1998) in the case of the His-containing peptides. Generally, the quenching of free radical has been attributed to the presence of specific amino acids (Byun, Lee, Park, Jeon, & Kim, 2009). The DPPH radical scavenging activities reduced with increasing peptide size and lower molecular weight peptides are more effective as strong DPPH radical scavengers than high molecular weight peptides. (Batista, Ramos, Coutinho, Bandarra, & Nunes, 2010).

ABTS radical scavenging activity

ABTS radical is comparatively stable but readily reduced by antioxidants. The scavenging activity against cationic ABTS[•] represent the ability of peptide fractions to act as electron donors or hydrogen donors in free radical reaction. In general, ABTS radical scavenging activities of protein hydrolysates increased as DH increased ($p < 0.05$) (Table 1). ESE showed a slight decrease in scavenging ability during the first 2 h of hydrolysis (3.37% decrease) which thereafter remained almost increased ($p < 0.05$).

The highest activity was observed in EBE and ESE with 44 and 49% DH ($p < 0.05$). Among all hydrolysates, EBA at 4h had the lowest activity for all DH tested ($p < 0.05$). Protein hydrolysate from alkali-solubilized tilapia protein prepared using various proteases showed a sharp increase in ABTS radical scavenging activity when DH increased from 18 to 23% (Raghavan, Kristinsson, & Leeuwenburgh, 2008). Fish protein hydrolysate showed a similar result, in which ABTS radical scavenging activity increased with increasing DH (You, Zhao, Cui, Zhao, & Yang, 2009). ABTS radical assay is used for determining the antioxidative activity, in which the radical is quenched to form ABTS-radical complex (Re et al., 1999). Generally, all hydrolysates contained peptides, which were able to scavenge ABTS radicals, leading to the termination of radical chain reaction. It was noted that the abilities of scavenging ABTS and DPPH radicals by hydrolysates were different. This might be due to the differences in ability of scavenging the different radicals, ABTS and DPPH, by the same protein.

Ferrous ion chelating activity

As shown in Table 2, the chelating ability of ESE and ESA decreased significantly in the first two of hydrolysis and then increased rapidly, whereas EBE and EBA possessed a increased after 2h of hydrolysis and then decreased thereafter. The ferrous chelating capacity of elastin extracted form both poultry skin gently decrease with increasing hydrolysis time. Interestingly, the result of spent hen skin displayed significantly ($p < 0.05$) higher metal chelating capacity than that of broiler at 5 mg/ml. Both enzyme used for hydrolysis showed a significant different Fe²⁺ ions activity: the most efficient was Elastase (71.71%) compared with Alcalase (59.68%).

Table-2: Effect of different enzymes and time of broiler and spent hen skin by chelating method in 5 (mg/ml) hydrolysis.

Time (hrs)	Broiler		Spent hen	
	Elastase	Alcalase	Elastase	Alcalase
2	69.60±0.12 ^d	52.59±0.17 ^b	73.40±0.06 ^b	64.98±0.17 ^b
4	69.41±0.16 ^{cd}	51.78±0.11 ^b	77.25±0.16 ^a	70.22±0.06 ^d
8	66.92±0.39 ^b	50.40±0.17 ^a	71.28±0.51 ^a	66.67±0.35 ^c
12	69.29±0.38 ^{cd}	52.58±0.88 ^b	72.21±0.73 ^{ab}	68.97±0.39 ^d
16	68.60±0.22 ^c	56.32±0.17 ^c	76.64±0.11 ^a	66.60±0.59 ^c
24	65.36±0.38 ^a	52.65±0.31 ^b	74.14±0.57 ^{ab}	62.49±0.59 ^a

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

Different lowercase in the column (for the same enzyme and different times) indicated significant difference ($p < 0.05$).

Ferrous ion (Fe²⁺) is the most potent pro-oxidant amid metal ions. This ion can combine with hydrogen peroxide in the Fenton reaction to produce reactive oxygen species and an hydroxyl free radical (OH), leading to the beginning and/or acceleration of lipid oxidation. The ferrous chelating activity by certain peptides in hydrolysates could tardy the oxidative reaction. Ferrozine slightly forms complexes with the Fe²⁺ ion. In the presence of chelating agents, the complex formation is interrupted, resulting in the reduction in colour formation (Thiansilakul, Benjakul, & Shahidi, 2007b).

CONCLUSION

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 antioxidative activities. Chicken skin is an economically and technologically viable substrate from which to extract elastin.

The antioxidative activities result was shown to be dependent on the source of the poultry skin (broiler and spent hen), type of enzymes used for hydrolysis (Alcalase and Elastase). Therefore, hydrolysate elastin from poultry skin could serve as a natural antioxidant for food preservation or as the functional foods. Further studies that involve peptide purification, amino sequencing of active peptides and in vivo studies, however need to be done to determine bioavailability.

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