# Enzymatic hydrolysis of ovomucoid and the functional properties of its hydrolysates

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**ABSTRACT** Ovomucoid is well known as a "trypsin inhibitor" and is considered to be the main food allergen in egg. However, the negative functions of ovomucoid can be eliminated if the protein is cut into small peptides. The objectives of this study were to hydrolyze ovomucoid using various enzyme combinations, and compare the functional properties of the hydrolysates. Purified ovomucoid was dissolved in distilled water (20 mg/mL) and treated with 1% of pepsin, $\alpha$-chymotrypsin, papain, and alcalase, singly or in combinations. Sodium sodium dodecyl sulfate-polyacrylamide (**SDS-PAGE**) results of the hydrolysates indicated that pepsin (OMP), alcalase (OMAl), alcalase + trypsin (OMAlTr), and alcalase + papain (OMAlPa) treatments best hydrolyzed the ovomucoid, and the 4 treatments were selected to determine their functional characteristics. Among the 4 enzyme treatments, hydrolysate from OMAlTr showed the highest iron-chelating and antioxidant activities, while OMP showed higher ACE-inhibitory activity, but lower Fe-chelating activity than the other treatments. However, no difference in the copper-chelating activity among the treatments was found. MS/MS analysis identified numerous peptides from the hydrolysates of OMAlPa and OMAlTr, and majority of the peptides produced were <2 kDa. Pepsin treatment (OMP), however, hydrolyzed ovomucoid almost completely and produced only amino acid monomers, di- and tri-peptides. The ACE-inhibitory, antioxidant and iron-chelating activities of the enzyme hydrolysates were not consistent with the number and size of peptides in the hydrolysates, but we do not have information about the quantity of each peptide present in the hydrolysates at this point.

**Key words:** Ovomucoid, enzyme hydrolysis, peptides, functional properties

2015 Poultry Science 94:2280–2287
http://dx.doi.org/10.3382/ps/pev196

## INTRODUCTION

Ovomucoid (11%) is one of the major functional protein found in egg white (Stadelman and Cotterill, 2001; Yousif and Kan, 2002). It is one of the most highly glycosylated proteins among the egg white proteins (Nolan et al., 2000). Also, it is well known as a "trypsin inhibitor" and is considered to be the main food allergen in egg. Ovomucoid is described as a single headed inhibitor of trypsin, meaning that each ovomucoid molecule binds 1:1 with trypsin, and its 3-dimensional structure is secured with its 3 disulfide bonds (Oliveria et al., 2009). Even though reported molecular weight of ovomucoid is 20.1 kDa, it is determined in Sodium sodium dodecyl sulfate-polyacrylamide (SDS-PAGE) to fall between 30 to 40 kDa. The pI value of the protein is 4.82 (Huopalahti et al., 2007). Hydrolyzed glycopeptides from ovomucoid using pepsin has immunoreactivity, but it is still uncertain whether the carbohydrates attached have any effect on this activity (Matsuda et al., 1985). Ovomucoid was first separated using trichloroacetic acid (**TCA**) and acetone by Lineweaver and Murray (1947) followed by a TCA and ethanol combination by Fredericq and Deutsch (1949), 3 step chromatography by (Davis et al., 1971), use of ethanol by (Tanabe et al., 2000), and SDS-PAGE with a linear gradient from 4 to 20% by (Yousif and Kan, 2002). Recently, ovomucoid was separated from egg white using a combination of ammonium sulfate and citric acid (Abeyrathne et al., 2013). Ovomucoid was separated easily from egg white and was highly soluble in water after freeze drying.

Egg white was hydrolyzed with alcalases enzymes to produce bio active peptides. Nolan et al. (2000) showed that peptides derived from ovomucoid using pepsin showed Immunoglobulin G (IgG)-binding

© 2015 Poultry Science Association Inc.
Received November 10, 2014.
Accepted May 29, 2015.
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activity and retained its trypsin inhibitory activities. The egg white was hydrolyzed with thermolysin produced bioactive peptides, which is a good Angiotensin-converting-enzyme (**ACE**) inhibitor, as well exhibiting antioxidant activity (Chiang et al., 2006). Recently Yu et al. (2012) reported the secondary structure of the ACE-inhibitory peptides. These authors have identified several peptides with ACE-inhibitory activity, which have the amino acid sequence of RVPSLM, TPSPR, DLQGK, AGLAPY, RVPSL, DHPFLF, HAEIN, QIGLF, HANENIF, VKELY and TNGIIR. Among them, TNGIIR showed the highest ACE-inhibitory activity. Other than hydrolyzing egg white as a whole, ovalbumin was used to produce bioactive peptides. Hydrolysates of ovalbumin showed strong ACE-inhibitory activities as well as reducing hypertension (Fujita et al., 2000; Miguel and Aleixandre, 2006; Manso et al., 2008). Moon et al. (2013) reported that peptides derived from ovotransferrin have cytotoxic effects on human cancer cells. Recent studies done in our laboratory showed that peptides derived from ovalbumin, as well as ovomucin, have many functional properties such as ACE-inhibitory, metal chelating, and antioxidant activities. However, no report on the production of functional peptides using ovomucoid or the functional properties of the peptides produced from ovomucoid are available. The objective of this study is to develop safe and simple ways of hydrolyzing ovomucoid to produce bioactive peptides that can be used for their antioxidant, metal chelating, and ACE-inhibitory properties.

## MATERIALS AND METHODS

### Materials

A total of 300 medium size fresh brown chicken eggs (less than 5-day-old) were purchased from the local market to produce ovomucoid. Standard enzymes pepsin, $\alpha$-chymotrypsin, trypsin, protease from *Bacillus licheniformis* (Alcalase® 2.4L), and papain were purchased from Sigma-Aldrich (St. Louis, MO). Other chemicals were purchased from Fisher Scientific (Thermo Fisher Scientific Inc., Waltham, MA). Ovomucoid was separated using a combination of ammonium sulfate and citric acid after extraction with ethanol (Abeyrathne et al., 2013). Egg white was diluted with the same volume of distilled water and $Fe^{3+}$ (0.8 ml/L of 500 mM $FeCl_3$) was added to convert ovotransferrin to "holo" form. Next, ethanol was added (43%, final concentration) to extract ovomucoid from the egg white and the resulting solution centrifuged. The supernatant was collected and the ethanol in the supernatant was removed using ultrafiltration (Hollow fiber, 10,000 cut-off size; Quickstand, GE healthcare Bio-Sciences Corp., Piscataway, NJ). The ovomucoid in the supernatant was separated by precipitating ovotransferrin and other impurities using a combination of 2.5% (w/v) ammonium sulfate and 2.5% (w/v) citric acid. After centrifugation, the supernatant was collected; the salts were removed using ultrafiltration, and the resulting mixture lyophilized (Labconco Corp., Kansas City, MO). The purity of ovomucoid used was 92%.

### Hydrolysis of Ovomucoid

Lyophilized ovomucoid (92% purity) was dissolved in distilled water at 20 mg/mL concentration (10 mL) and the pH was adjusted using 1N HCl or 1N NaOH depending on the optimal conditions for each enzyme: pepsin (pH 2.5, 37°C), trypsin (pH 7.8, 37°C), $\alpha$-chymotrypsin (pH 7.6, 37°C), alcalase (pH 6.5, 55°C), and papain (pH 6.5, 37°C). Pepsin, trypsin, $\alpha$-chymotrypsin, papain, and alcalase were added singly or in combination (2 enzymes in sequence) (enzyme: substrate = 1:100), and incubated for 0, 3, 6, 9, 12, and 24 hours. At the end of incubation, the samples were heated at 100°C for 15 min to inactivate the added enzyme.

### SDS-PAGE

SDS-PAGE was used to analyze the level of hydrolysis after treating ovomucoid with the enzymes. Fifteen percent gels were prepared, and SDS-PAGE was conducted under reducing conditions using a Mini-Protein II cell (Bio-Rad, Hercules, CA). Fifteen percent SDS gel and Coomassie Brilliant Blue R-250 (Sigma) staining were used (Price and Nairn, 2009).

### Measurement of Functional Properties

In order to measure the functional properties of the hydrolyzed products from ovomucoid, the 4 best enzyme treatments that hydrolyzed ovomucoid to small peptides were selected after analyzing the SDS-PAGE pictures and the physical appearance of the products after hydrolysis.

***Antioxidant Activity.*** The antioxidant activity of the hydrolyzed ovomucoid was measured using the method of Abeyrathne et al. (2014) with some modifications. An oil-in-water emulsion was prepared by homogenizing 1.0 g of corn oil (Ottogi Company, Seoul, Korea) and 100 $\mu$L of Tween 20 with 100 mL of distilled water using a polytron (Brinkman Instruments Inc., Westbury, NY, USA) for 2 min in an ice bath at full power. Samples for lipid oxidation assay were prepared by mixing 8 mL of oil emulsion, 0.5 mL of 0.2% ascorbic acid (Sigma-Aldrich, St. Louis, MO, USA), 0.5 mL of 200 ppm $Fe^{2+}$ ($FeSO_4$, Sigma) and 1 mL of hydrolyzed products of ovomucoid. The samples were then incubated at 37°C for 16 hr. At the end of incubation, 1 mL of sample was transferred to a 15 mL falcon tube, and 2 mL of thiobarbituric acid/trichloroacetic acid solution (20 mM TBA/15% TCA) (w/v) and 50 $\mu$L of 10% butylated hydroxyanisole in 90% ethanol were added and the resulting fluid vortex-mixed. The mixture was incubated in a 90°C water bath for 15 min

to develop color. The sample was then cooled in an ice bath for 10 min and centrifuged at 3,000 × $g$ for 15 min at 5°C. The absorbance of the solution was measured at 532 nm against a blank prepared with 1 mL of distilled water and 2 mL TBA/TCA solution. The amounts of 2-thiobarbituric acid reactive substances (**TBARS**) were expressed as mg of malondialdehyde (MDA) per L of emulsion.

***$Fe^{2\pm}$-Chelating Activity.*** The Fe-chelating activity of the hydrolysates of ovomucoid was measured using the ferrozine method (Carter, 1971) with some modifications. One hundred $\mu$L of the hydrolysates, 0.9 mL of distilled water and 1 mL of 10 ppm $Fe^{2+}$ ($FeSO_4$, Sigma) were vortex-mixed in a 15 mL Falcon tube and incubated for 5 min at room temperature. To remove the proteins and peptides in the sample, 900 $\mu$L of 11.3% TCA was added and then centrifuged at 2,500 × $g$ for 10 min. One mL of the supernatant was transferred to a disposable culture tube, and 1 mL of distilled water, 800 $\mu$L of 10% ammonium acetate (Thermo Fisher Scientific Inc., Waltham, MA, USA), and 200 $\mu$L of ferroin color indicator (75 mg of ferrozine, 75 mg of neocuprion and 1 drop of 6 N HCl in 25 mL of distilled water) were added, and the results vortex-mixed. After 5 min of incubation at room temperature, the absorbance was measured at 562 nm against a blank prepared with 2 mL of distilled water, 800 $\mu$L of 10% ammonium acetate and 200 $\mu$L of ferroin color indicator for iron-chelating activity. The Fe chelating activity was calculated using the following equation:

$$\text{Fe chelating activity }\% = (1 - \text{sample absorbance}/\text{blank absorbance}) \times 100$$

***$Cu^{2\pm}$-Chelating Activity.*** The Cu-chelating activity of the hydrolyzed ovomucoid was measured using the method of Kong and Xiong (2006) with some modifications. One mL of 0.2 mM $CuSO_4$ was mixed with 1 mL of hydrolysate in a 15 mL Falcon tube and incubated for 5 min at room temperature. After adding 1 mL of 11.3% TCA solution, the sample was centrifuged at 2,500 × $g$ for 10 min. Two mL of the supernatant was transferred to a disposable culture tube and then 1 mL of 10% pyridine and 20 $\mu$L of 0.1% pyrocatechol violet (Sigma-Aldrich, St. Louis, MO, USA) were added. The resulting mixture was vortex-mixed and incubated for 5 min at room temperature. The absorbance of the samples were measured at 632 nm against a blank prepared with 2 mL of Distilled water (**DW**), 1 mL of 10% pyridine, and 20 $\mu$L of 0.1% pyrocatechol violet. The chelating activity was measured using the following equation:

$$\text{Cu chelating activity }\% = \{1 - (\text{sample absorbance}/\text{blank absorbance})\} \times 100$$

***ACE-inhibitory Activity.*** ACE-inhibitory activity of the hydrolysates of ovomucoid was measured using the methods of Miguel et al. (2007) and Yu et al. (2012) with some modifications. An aliquot of sample (40 $\mu$L) was incubated with 100 $\mu$L of 0.1M borate buffer (pH 8.3) containing 5 mM of Hippuryl-Histidil-Leucine, 0.3 M NaCl, and 20 $\mu$L of ACE (0.1 U) at 37°C for 30 min. After incubation, the reaction was stopped by adding 150 $\mu$L of 1 M HCl. The hippuric acid formed was extracted with 1,000 $\mu$L of ethyl acetate and centrifuged at 1,500 × $g$ for 10 min. Next, 750 $\mu$L of the organic phase was separated and, using a dry heat block, the organic phase was evaporated at 95°C for 10 min and then 800 $\mu$L of distilled water was added and the resulting fluid vortex mixed. Absorbance was measured at 228 nm against a control prepared with 40 $\mu$L of distilled water. ACE-inhibitory activity was measured using the following equation.

$$\text{ACE - inhibitory activity} = \{1 - (\text{blank abs} - \text{sample abs})/\text{blank abs}\} \times 100$$

***Identification of Peptides in the Hydrolysates.*** A Dionex U 3000 HPLC system equipped with a Thermo-Finnigan LTQ-Orbitrap (ion trap with a nano-electrospray ionization source, Thermo Fisher Scientific Inc., Waltham, MA, USA) mass spectrometer was used to identify the peptides in the enzyme hydrolysates. Fractions from HPLC were reconstituted in solvent A (water/aetonitrile = 98:2, v/v, 0.1% formic acid) and then injected into a LC-nano ESI-MS/MS system (Thermo Fisher Scientific Inc., Waltham, MA, USA). Samples were first trapped on a Zorbax 300SB-C18 trap column (300 $\mu$m i.d × 5 mm, 5 $\mu$m, 100 Å (Agilent Technologies, Wilmington, DE, USA), part number 5065—9913) and washed for 6 min with 98% solvent A (water/aetonitrile = 98:2, v/v; 0.1% formic acid) and 2% solvent B (Water/aetonitrile = 2:98, v/v; 0.1% formic acid) at a flow rate of 5 $\mu$L/min, and then separated on a Zorbax 300SB-C18 capillary column (75 $\mu$m i.d x 150 mm, 3.5 $\mu$m, 100 Å, part number 5065–9911) at a flow rate of 300 nL/min. The liquid chromatography (**LC**) gradient was run at 2% to 35% solvent B over 30 min, then from 35% to 90% over 10 min, followed by 90% solvent B for 5 min, and finally 5% solvent B for 15 min. Resulting peptides were electrosprayed through a coated silica tip (FS360–20–10-N20-C12, PicoTip emitter, New Objective, Inc., Woburn, MA, USA) at an ion spray voltage of 2,000 eV. Mass data were acquired over the mass range of 400 to 5,000 Da using a proteome discoverer (Thermo-Finnigan, San Jose, CA, USA).

## Statistical Analysis

Data were analyzed with MINITAB16.0 statistical software. One way ANOVA was used and LSD tests were performed for the significant differences ($P < 0.05$) among means. All trials were replicated 3 times.

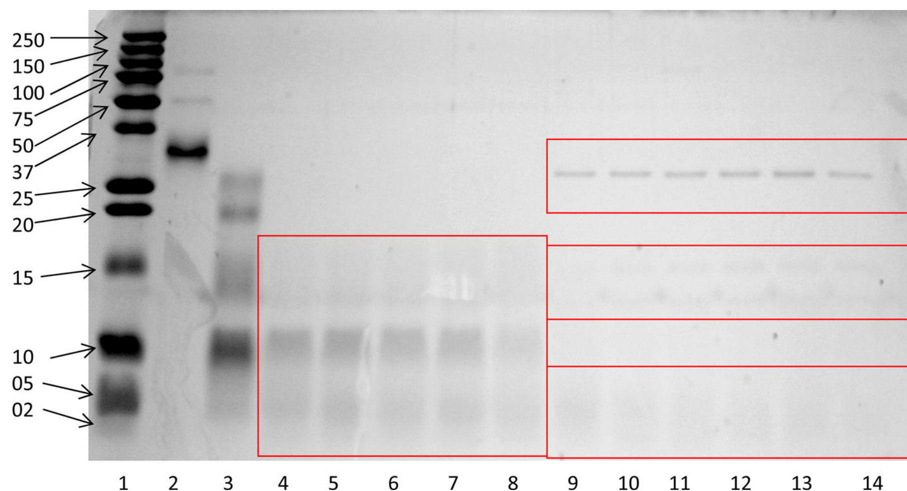


**Figure 1.** SDS-PAGE of ovomucoid hydrolyzed with pepsin (OMP) and alcalase (OMAl) as a single enzyme. Lane 1 = Marker, Lane 2 = diluted ovomucoid (1 mg/mL), Lanes 3 to 8 = hydrolyzed ovomucoid with pepsin incubated at 37°C for 0, 3, 6, 9, 12, and 24 hr, Lanes 9 to 14 = hydrolyzed ovomucoid with alcalase incubated at 55°C for 0, 3, 6, 9, 12, and 24 hr.

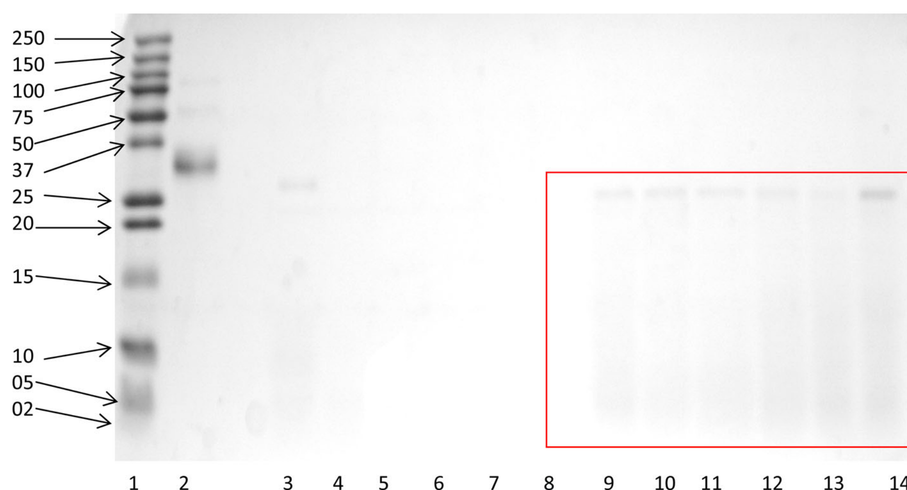


**Figure 2.** SDS-PAGE of ovomucoid hydrolyzed with alcalase, and then with trypsin (OMAlTr) or papain (OMAlPa). Lane 1 = Marker, Lane 2 = diluted ovomucoid (1 mg/mL), Lanes 3 to 8 = hydrolyzed ovomucoid with alcalase (55°C) followed by trypsin (37°C) for 0, 3, 6, 9, 12, and 24 hr, Lanes 9 to 14 = hydrolyzed ovomucoid with alcalase (55°C) followed (37°C) by papain at 37°C for 0, 3, 6, 9, 12, and 24 hrs.

## RESULTS AND DISCUSSION

### Hydrolysis of Ovomucoid

Ovomucoid was almost completely hydrolyzed with pepsin and alcalase (Figure 1, the faint remaining peptide bands can be seen in the boxes), but $\alpha$-chymotrypsin was not effective (data not shown). Papain partially hydrolyzed ovomucoid, but was not as effective as pepsin and alcalase (data not shown). Trypsin was not used as the primary enzyme due to its inhibitory activity. The effect of 2-enzyme combinations on hydrolysis of ovomucoid indicated that 1% alcalase treatment for 3 hr at 55°C followed by 1% trypsin or 1% papain treatment at 37°C for 3 hr produced a smaller size of peptide than those with 1% alcalase alone (Figure 2). However, 1% pepsin treatment for 3 hr followed by 1% trypsin or 1% papain treatment did not improve the hydrolysis (data not shown). Therefore, combinations of these 2 enzymes with pepsin are not recommended. Among the treatments, pepsin at 37°C for 3 hr (**OMP**), alcalase at 55°C for 3 hr (**OMAl**), alcalase at 55°C for 3 hr with trypsin at 37°C for 3 hr (**OMAlTr**), and alcalase at 55°C for 3 hr with papain at 37°C for 3 hr (**OMAlPa**) were the best in hydrolyzing ovomucoid and these 4 treatments were used to analyze metal-chelating, antioxidant, and ACE-inhibitory activities as the functional properties.

### Functional Activity of Ovomucin Hydrolysates

***Antioxidant Activity.*** Figure 3 indicated that the ovomucoid hydrolysates of OMP, OMAl, OMAlTr, and OMAlPa showed strong antioxidant activity. The hydrolysates of these 4 treatments reduced the TBARS value of oil emulsion by about 70 to 80% compared to the control, indicating that the treatments produced peptides with strong antioxidant properties.

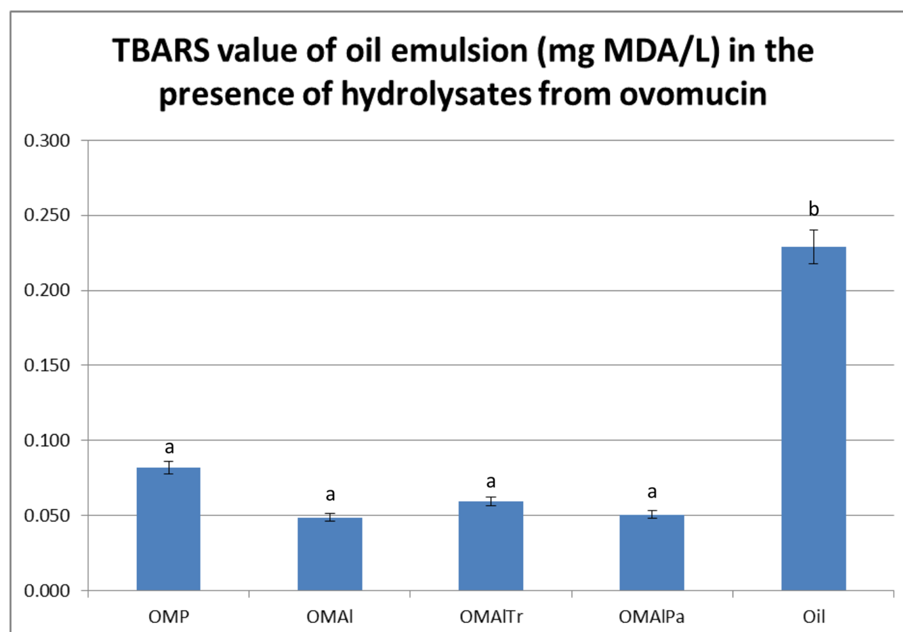


**Figure 3.** TBARS value of oil emulsion (mg MDA/L) in the presence of the hydrolysates from ovomucoid (OMP = ovomucoid hydrolyzed with 1% pepsin for pepsin for 3 hr at 37°C, OMAl = ovomucoid hydrolyzed with 1% alcalase for 3 hr at 55°C, OMAlTr = ovomucoid hydrolyzed with 1% alcalase for 3 hr at 55°C followed by 1% trypsin for 3 hr at 37°C, OMAlPa = ovomucoid hydrolyzed with 1% alcalase for 3 hr at 55°C followed by 1% papain for 3 hr at 37°C). Values are mean with standard error. Values with different letters are significantly different ($P < 0.05$).

Ovomucoid is well known for its trypsin inhibitory activity and as a food allergen (Huopalahti et al., 2007), but no report on antioxidant effect of ovomucoid or its hydrolysates are available. Davalos et al. (2004) reported that enzymatic hydrolysis of egg white produced peptides with antioxidant and free radical scavenging activities, and that the peptide with the strongest antioxidant activity had amino acid sequence of Tyr-Ala-Glu-Glu-Arg-Tyr-Pro-Ile-Leu. Zambrowicz et al. (2012) also reported that not only the hydrolysates of egg white proteins, but also those of yolk proteins, have strong antioxidant activity. Among the egg white proteins, only ovotransferrin shows antioxidant activity (Stadelman and Cotterill, 2001). However, hydrolysates of ovalbumin (Lin et al., 2011, Abeyrathne et al., 2014), ovomucin (Chang et al., 2013), lysozyme, and cystatin (Graszkiewicz et al., 2007) are reported to have antioxidant effects.

***Metal-chelating Activity.*** Figure 4A indicated that ovomucoid hydrolyzed with combination of alcalase plus trypsin (OMAlTr) showed the strongest iron-chelating activity ($P < 0.05$) among the 4 selected treatments: OMAl and OMAlPa showed similar levels (~75%) of iron-chelating activity compared to OMAlTr, but OMP had the lowest iron-chelating activity among the treatments (Figure 4A). Ovotransferrin was reported to have a strong iron chelating activity (Ko and Ahn, 2008), but ovomucoid or its hydrolysates have never been reported as having metal-chelating activity. The peptides produced with the combination of alcalase plus trypsin showed very strong iron-chelating activity. Huopalahti et al. (2007) reported that peptides with high iron-chelating activity can prevent oxidation. Microbial growth can also be reduced by peptides with strong iron-chelating activities (Ko et al., 2009), and thus these peptides can be used as antimicrobial agents for foods.

Figure 4B indicated that the ovomucoid hydrolysates from all 4 treatments showed 60 to 70% $Cu^{2+}$-chelating activities. Previous work done in our laboratory showed that the hydrolysates of ovalbumin prepared with pepsin at 37°C for 3 hr, pepsin at 37°C for 3 hr plus $\alpha$- chymotrypsin at 37°C for 3 hr, pepsin at 37°C for 3 hr plus alcalase at 55°C for 3 hr, pepsin at 37°C for 3 hr plus papain at 37°C for 3 hr, and alcalase at 55°C for 3 hr plus trypsin at 37°C for 3 hr had similar $Cu^{2+}$-chelating activities (Abeyrathne et al., 2014).

***ACE-inhibitory Activity.*** Figure 5 indicated that ovomucoid hydrolysates produced by pepsin showed the highest ACE-inhibitory activity among the 4 treatments even though the other 3 treatments also showed 60 to 70% ACE-inhibitory activity. Previous research has indicated that enzymatic hydrolysis of egg white protein, as well as ovalbumin, produced peptides with strong ACE-inhibitory activities (Chiang et al., 2006; Miguel et al., 2006; Miguel et al., 2007). However, no previous report on the ACE-inhibitory activity of ovomucoid or its hydrolysates are available. Miguel and Alexiandre (2006) reported that the peptides with the amino acid sequence Tyr-Arg-Glu-Glu-Arg-Tyr-Pro-Ile-Leu; Arg-Ala-Asp-His-Pro-Phe-Leu and Ile-Val-Phe have strong ACE-inhibitory activities. Miguel et al. (2007) showed that peptides derived from egg white proteins using pepsin have antihypertensive, ACE-inhibitory, and vasodilator properties. Egg white hydrolysates reduced oxidative stress and controlled blood

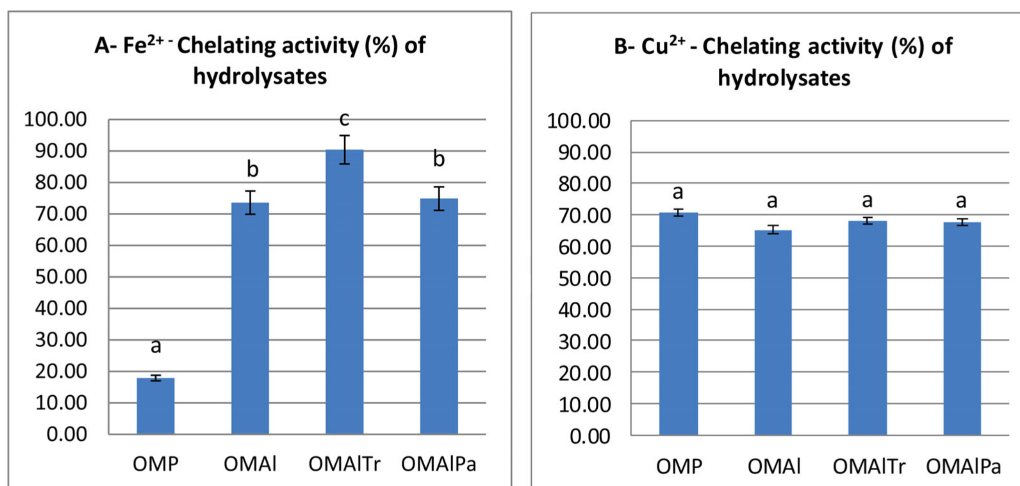


**Figure 4.** Graphical expression of $Fe^{2+}$- and $Cu^{2+}$-chelating activities of the hydrolysates from ovomucoid (OMP = ovomucoid hydrolyzed with 1% pepsin for 3 hr at 37°C, OMAl = ovomucoid hydrolyzed with 1% alcalase for 3 hr at 55°C, OMAlTr = ovomucoid hydrolyzed with 1% alcalase for 3 hr at 55°C followed by 1% trypsin for 3 hr at 37°C, OMAlPa = ovomucoid hydrolyzed with 1% alcalase for 3 hr at 55°C followed by 1% papain for 3 hr at 37°C). Values are mean with standard error. Values with different letters are significantly different ($P < 0.05$).

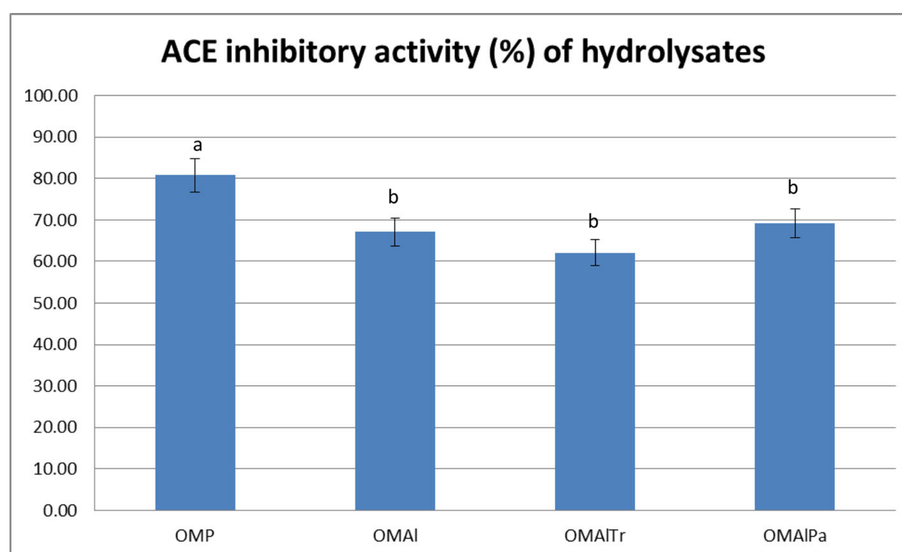


**Figure 5.** Graphical expression of ACE-inhibitory activity of the hydrolysates from ovomucoid (OMP = ovomucoid hydrolyzed with 1% pepsin for 3 hr at 37°C, OMAl = ovomucoid hydrolyzed with 1% alcalase for 3 hr at 55°C, OMAlTr = ovomucoid hydrolyzed with 1% alcalase for 3 hr at 55°C followed by 1% trypsin for 3 hr at 37°C, OMAlPa = ovomucoid hydrolyzed with 1% alcalase for 3 hr at 55°C followed by 1% papain for 3 hr at 37°C). Values are mean with standard error. Values with different letters are significantly different ($P < 0.05$).

lipid profile on spontaneously hypertensive rats (Manso et al., 2008). Therefore, the peptides derived from ovomucoid also can be used as ACE-inhibitory, blood pressure-lowering, and antioxidant agents in the animal body. OMP is good for ACE-inhibitory activity and OMAlTr, OMAl, OMAlPa have excellent metal chelating and antioxidant effects.

## Identification of Peptides in the Hydrolysates

The LC-nano ESI-MS/MS potentially identified 72 peptides with a molecular weight range of 500 to 5,000 Da from the alcalase hydrolysate (OMAl), 35 from the pepsin (OMP), 170 from alkalase plus papain (OMAlPa), and 75 from alcalase plus trypsin (OMAlTr). More than half of the peptides from OMAl (44 out of 72), OMAlPa (117 out of 170), and OMAlTr (46 out of 75) had a molecular weight smaller than 2 kDa. However, no detectable peptides from ovomucoid were found in the pepsin (OMP) hydrolysates. All the peptides detected from the pepsin hydrolysate were from impurities such as flavoprotein, ovotransferrin, and ovoinhibitor. This indicated that pepsin hydrolyzed ovomucoid almost completely and produced only amino acid monomers, di- and tri-peptides (MW < 400) (Table 1).

All the enzyme hydrolysates had excellent antioxidant and ACE-inhibitory activities, but the Fe-chelating capacity of the hydrolysates differ among enzyme treatments: pepsin treatment (OMP) had the

**Table 1.** Effects of enzyme treatments on the size and number of peptides produced from ovomucoid.[1]

| MW of peptides (Da) | Enzyme treatments OMAl | OMP | OMAlPa | OMAlTr |
|---|---|---|---|---|
| 500–1,000 | 10 | 0 | 25 | 10 |
| 1,000–1,500 | 14 | 0 | 53 | 15 |
| 1,500—2,000 | 20 | 0 | 39 | 21 |
| 2,000–3,000 | 20 | 0 | 44 | 19 |
| 3,000–5,000 | 8 | 0 | 9 | 10 |
| Total (ProtScores) | 72 (6.90) | 0 | 170 (>1.24) | 75 (6.90) |

[1]Peptides are identified using a LC-nano ESI-MS/MS method. Peptides with ProtScores > 2.0, > 1.3, and > 0.47 have > 99%, > 95%, and > 66% probability, respectively.

Treatments: OMP = ovomucoid hydrolyzed with 1% pepsin for 3 hr at 37°C; OMAl = ovomucoid hydrolyzed with 1% alcalase for 3 hr at 55°C; OMAlTr = ovomucoid hydrolyzed with 1% alcalase for 3 hr at 55°C followed by 1% trypsin for 3 hr at 37°C, OMAlPa = ovomucoid hydrolyzed with 1% alcalase for 3 hr at 55°C followed by 1% papain for 3 hr at 37°C.

lowest, alcalase (OMAl) and alcalase plus papain (OMAlPa) treatments had intermediate, and alcalase plus trypsin (OMAlTr) had the highest Fe-chelating capacity (Figures 3 and 4). The ACE-inhibitory, antioxidant and iron-chelating activities of the enzyme hydrolysates (Figures 3–5) were not consistent with the number and size of peptides in the hydrolysates. However, the low Fe-chelating activity of pepsin hydrolysate indicated that pepsin is not a good enzyme for the production of peptides with iron-chelating functions from ovomucoid. Currently however, we do not have information about the amount of each peptide present in the hydrolysates.

## CONCLUSION

Ovomucoid (with 92% purity) could be hydrolyzed easily and produced bioactive peptides. Among them, hydrolysates produced from OMAlTr treatment showed the highest iron-chelating and antioxidant activities. Copper-chelating activity among the treatments was the same, while OMP treatment produced peptides with the highest ACE-inhibitory activity, but with the lowest iron-chelating and antioxidant activities. The peptides derived from the ovomucoid (92% purity) using these enzyme treatments can be used as nutraceutical agents or in food processing.

## ACKNOWLEDGMENTS

This study was supported by the Cooperative Research Program for Agriculture Science & Technology Development (Project No. PJ01128901), Rural Development Administration, Republic of Korea.

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