Enzymatic Hydrolysis of Ovomucin and the Functional and Structural Characteristics of Peptides in the Hydrolysates

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Summary and Implications

Ovomucin was hydrolyzed using enzymes or by heating under alkaline conditions (pH 12.0), and the functional, structural and compositional characteristics of the peptides in the hydrolysates were determined. Among the treatments, heating at 100 ºC for 15 minutes under alkaline conditions (OM) produced peptides with the highest iron-binding and antioxidant capacities. Ovomucin hydrolyzed with papain (OMPa) or alcalase (OMAl) produced peptides with high ACE-inhibitory activity. The mass spectrometry analysis indicated that most of the peptides from OMPa were < 2 kDa, but peptides from OMTr and OM were > 2 kDa. OMAl hydrolyzed ovomucin almost completely and no peptides within 700-5,000 Da were found in the hydrolasate. The results indicated that the number and size of peptides were closely related to the functionality of the hydrolysates. Considering the time, cost and activities of the hydrolysates, OM was the best treatment for hydrolyzing ovomucin to produce functional peptides.

Introduction

Ovomucin is a sulfated glycoprotein with molecular weight ranges from 150 kDa to 23,000 kDa and is responsible for the gel-like structure of thick in egg albumin. Enzymatic hydrolysis is one of the methods to improve functional properties of ovomucin. Hydrolysis of ovomucin with Neutraese, Flavourzyme, Alcalase, or Pronase E produced peptides with strong foaming capacities, but had low foam stabilities. The pronase hydrolysis of ovomucin produced an ovomucin glycopeptide that has *E. coli* O157:H7-specific binding sites, and suggested that this peptide could be used as an antimicrobial agent against *E. coli* O157:H7 and to be a novel probe for the detection of *E. coli* O157:H7 in the foods. The hydrolysates of ovomucin had strong antioxidant effects. The objectives of this study were 1) to develop a simple and easy way of hydrolyzing ovomucin, 2) to determine the functional characteristics (antioxidant, metal chelating, and ACE-inhibitory) of the peptides in the hydrolysates, and 3) to elucidate the compositional and structural characteristic of the peptides in the hydrolysates.

Materials and Methods

The lyophilized ovomucin was dissolved in distilled water at 20 mg/ml concentration and used in this study. The pH of the ovomucin solution was adjusted for the optimal conditions for each enzyme (pepsin pH 2.5, trypsin pH 7.8, papain pH 6.5 and alcalase pH 6.5) under room temperature for optimal enzyme hydrolysis. Pepsin, trypsin, or papain were dissolved in distilled water (20 mg enzyme/ml) before use, but Alcalase® 2.4L was in aqueous form and used as is. Each enzyme was added to ovomucin solution (enzyme: substrate ratio at 1:100) and incubated at 37 ºC for 0, 3, 6, 9, 12 and 24 hr. At the end of incubation, the samples were heated at 100 ºC for 15 min to inactivate the enzyme. Also, hydrolysis of ovomucin by heating (100 ºC for 15 min) under alkaline conditions (pH 12.0) was tested. SDS-PAGE gels (15%) were used to analyze the peptides derived from ovomucin. Antioxidant, ACE-inhibitory, Fe-chelating and Cu-chelating activity of the hydrolyzed ovomucin were also measured. The peptides in the hydrolysates were determined using a LC-nano ESI-MS/MS system. Data were analyzed with MINITAB16.0 (Minitab Ltd., UK) statistical software. One way ANOVA was used: Least significance difference (LSD) tests were performed for the significant differences ($P < 0.05$) among means. All trials were replicated three times.

Results and Discussion

The lyophilized ovomucin was very difficult to dissolve in water under neutral or acidic pH conditions. However, the ovomucin was easily solubilized at pH > 12.0, and the majority was hydrolyzed by heating at 100 ºC for 15 min (Figure 1). Increasing heating time longer than 15 min did not increase hydrolysis but turned the color of ovomucin into brown probably due to caramelization reaction of carbohydrate units in the protein. Incubating ovomucin with 1% pepsin at 37 ºC for 24 h changed the ovomucin solution into a gel-like conditions even though the hydrolysis proceeded well (Figure 2). Treating ovomucin with trypsin produced small peptides (Figure 2), but the hydrolysis was not as complete as that of alcalase treatment (Figure 3). Treating ovomucin with 1% papain for 3 h also hydrolyzed ovomucin well (Figure 3), even though SDS-PAGE does not show any difference between 0 h and 3 h treatments. One difference between the 0 h and 3 h hydrolysis using 1% papain was that 0 h hydrolysis of ovomucin with papain produced precipitated proteins after heat inactivation of the enzyme. Therefore, incubating for 3 h at 37 ºC was selected as the optimum time and temperature combination for papain. According to the results, four treatments were selected as the best treatments for hydrolyzing ovomucin. The four selected treatments include heating ovomucin

solution at pH 12.0 for 15 minutes at 100 ºC (OM), hydrolyzing ovomucin with 1% trypsin at 37 ºC for 3 h (OMTr), hydrolyzing ovomucin with 1% alcalase at 37 ºC for 3 h (OMAl), and hydrolyzing ovomucin with 1% papain at 37 ºC for 3 h (OMPa). These four treatments were used to analyze their metal-chelating, antioxidant and ACE-inhibitory activities.

Ovomucin hydrolyzed by heating at 100 ºC for 15 minutes under pH 12.0 (OM) had a strong antioxidant activity (Figure 4A). The TBARS value of oil emulsion added with the hydrolysates was only 1/3 of the control. However, hydrolysates from other treatments increased the TBARS value of oil emulsion, indicating that they have strong prooxidant effects. The antioxidant activity of peptides from ovomucoid varied greatly depending upon the enzymes or conditions used to hydrolyze it. Hydrolysates from OMPa and OMAl treatments showed the highest ACE-inhibitory activity (> 70%) followed by OMTr with 65% inhibition (Figure 4B). However, the hydrolysates from OM treatment did not show a strong ACE-inhibitory activity. Therefore, ovomucin hydrolyzed with 1% alcalase (OMAl) and ovomucin hydrolyzed with 1% papain (OMPa) can be considered as good treatments to produce peptides with ACE-inhibitory activity. The results indicated that OMPa, OMAl, and OMTr treatments can be used to produce functional peptides with ACE-inhibitory activities. Figure 5A indicated that ovomucin hydrolyzed by heating at 100 ºC for 15 minutes under pH 12.0 (OM) showed a high iron-chelating activity ($P < 0.05$). OMTr showed some iron-chelating activity, but other two treatments (OMAl and OMPa) showed very low iron-chelating activities. It was observed that ovomucin had a capability of controlling bacteria such as *Escherichia coli*, *Bacillus dysentericus*, and *Vibrio bacterium*. With a high iron-chelating activity, microbial growth can be reduced. Therefore, these peptides can be used as antimicrobial agents for foods. Because the peptides produced from ovomucin through alkaline hydrolysis followed by heat treatment (OM) showed a high iron-chelating activity, the hydrolysates from OM treatment can be helpful in preventing oxidative changes and microbial growth in foods. The Cu^{2+}-chelating activity of the peptides from ovomucin was totally different from that of the iron-chelating activity. It was observed that the levels of Cu^{2+} ions in samples with the hydrolyzed products were higher than that of the blank, indicating that cupper ions were not chelated by the peptides from OMTr, OMAl and OMPa treatments (Figure 5B). Therefore, hydrolysates from OMTr, OMAl and OMPa are not suitable as antioxidant or metal chelating agents.

The LC-nano ESI-MS/MS potentially identified 642 peptides with the molecular weight range of 700-5,000 Da from the OMTr treatment, 182 from the OMPe hydrolysate, and 149 from the OM. The majority (173 out of 182) of the peptides from OMPa was smaller than 2 kDa, but more than half of the peptides produced from OMTr (371 out of 642 peptides) and OM (88 out of 149 peptides) was greater than 2 kDa. No detectable peptides from ovomucin were found in the OMAl hydrolasate. This indicated that treating ovomucin with 1% alcalase for > 3 hr hydrolyzed ovomucin almost completely to produce amino acid monomers, and peptides with MW < 700 Da (Figure 3 and Table 1). The high ACE-inhibitory activity of OMAl treatment can be related to the small peptide sizes, but it cannot be confirmed in this study. OMPa treatment produced smaller peptides than OM and OMTr treatments (Table 1).

The ACE-inhibitory, antioxidant and iron-chelating activities of the enzyme hydrolysates (Figures 4-5) indicated that the number (also amount) and size of peptides were closely related to the functionality of the hydrolysates: as the protein became hydrolyzed to smaller peptides, the antioxidant and Fe-chelating activities decreased while the ACE-inhibitory activity increased. At this point, however, no information about the amount of each peptide present in the hydrolysates is available. It is highly unlikely that certain functional characteristics of enzyme hydrolysates are derived only by one or two specific peptides (out of hundreds of peptides), unless their amounts are very high. Therefore, finding enzyme treatments that produce group of peptides with specific functional characteristics is the first step. Production of peptides that share similar functions from a protein and using the whole hydrolysate as a food ingredient or nutraceutical agents would be more reasonable and economical than identifying specific peptides and separating them from a hydrolysate before use.

Conclusion

The functions of hydrolysates can vary significantly depending upon the hydrolysis conditions and methods. Hydrolysate of OM treatment is suitable for metal-chelating and antimicrobial agents, while those of OMPa, OMAl and OMTr can be used for ACE-inhibitory agents. Production of these peptides is simple and can be easily used in the industrial level. Considering the time, cost and activities of the hydrolysates, OM treatment was the best for hydrolyzing ovomucin ($P < 0.05$) to produce functional peptides.

Figure 1: SDS-PAGE of ovomucin hydrolyzed with heat treatment at different temperatures for 15 min at pH 12.0. Lane 1 = Marker, Lane 2 = ovomucin (1 mg/ml), Lane 3 = ovomucin at 60 ºC for 15 min, Lane 4 = ovomucin at 70 ºC for 15 min, Lane 5 = ovomucin at 80 ºC for 15 min, Lane 6 = ovomucin at 90 ºC for 15 min, Lane 7 = ovomucin at 100 ºC for 15 min.

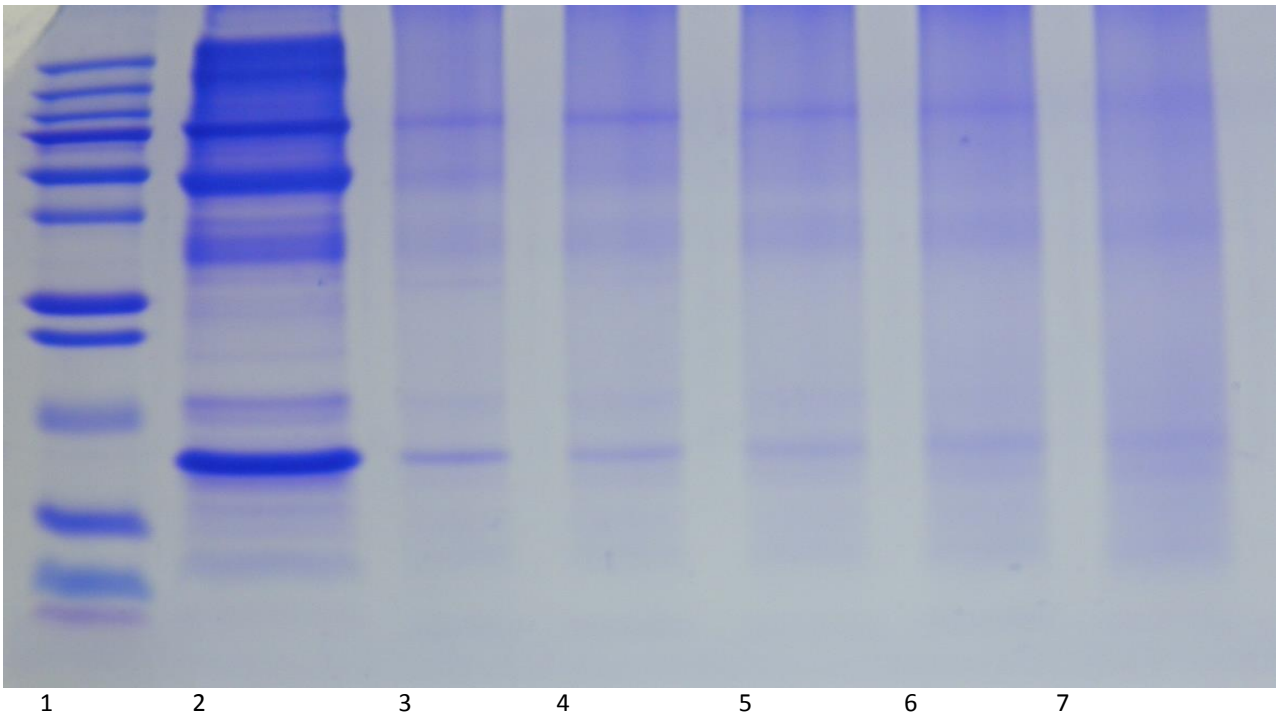

Figure 2: SDS-PAGE of ovomucin hydrolyzed with pepsin and trypsin. Lane 1 = Marker, Lane 2 = ovomucin (1 mg/ml), Lanes 3-8 = ovomucin hydrolyzed with pepsin at 37 ºC for 0, 3, 6, 9, 12 and 24 h, Lanes 9-14 = ovomucin hydrolyzed with trypsin at 37 ºC for 0, 3, 6, 9, 12 and 24 h.

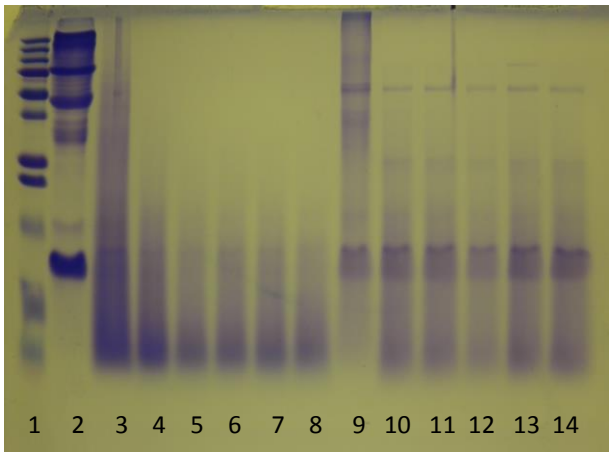

Figure 3: SDS-PAGE of ovomucin hydrolyzed with alcalase and papain. Lane 1 = Marker, Lane 2 = ovomucin (1 mg/ml), Lanes 3-8 = ovomucin hydrolyzed with alcalase at 37 ºC for 0, 3, 6, 9, 12 and 24 h , Lanes 9-14 = ovomucin hydrolyzed with papain at 37 ºC for 0, 3, 6, 9, 12 and 24 h.

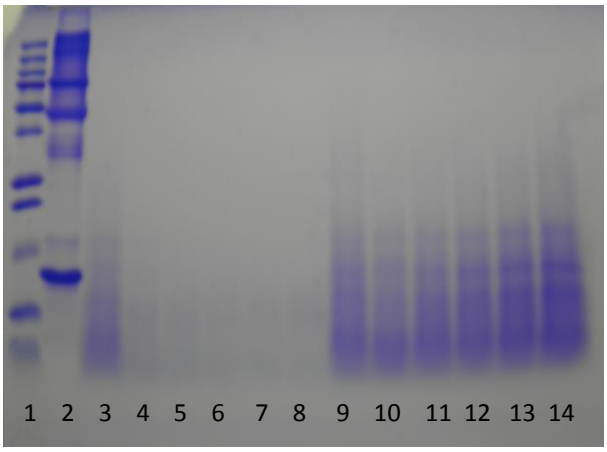

Figure 4. **A:** TBARS value of oil emulsion (mg MDA/L) in the presence of the hydrolysates from ovomucin, and **B:** ACE-inhibitory activity (%) of the hydrolysates from ovomucin (OM = ovomucin hydrolyzed with heating at 100 °C for 15 min at pH 12.0, OMT = ovomucin dissolved at pH 12.0 and hydrolyzed with 1% trypsin, OMPa = ovomucin dissolved at pH 12.0 and hydrolyzed with 1% papain, OMAl = ovomucin dissolved at pH 12.0 and hydrolyzed with 1% alcalase). [a-c]Values are mean with standard error. Values with different letters are significantly different ($P < 0.05$).

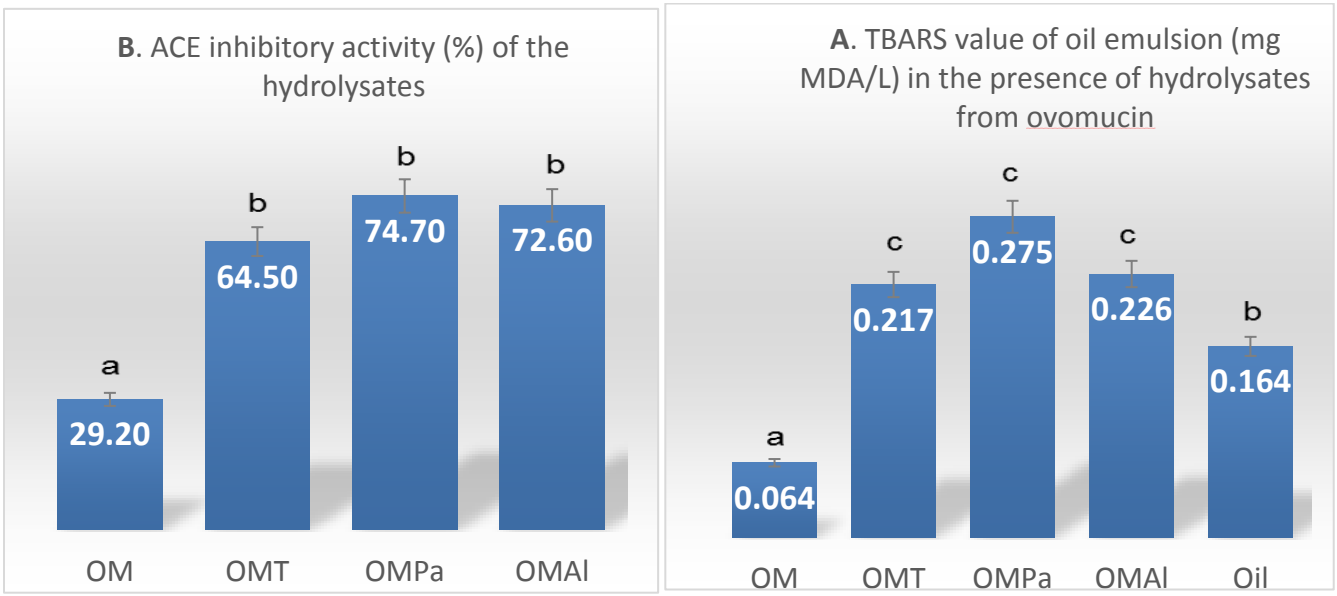

Figure 5: Graphical expression of Fe^{2+}- and Cu^{2+}-chelating activity of the hydrolysates from ovomucin (OM = ovomucin hydrolyzed with heating at 100 °C for 15 min at pH 12.0, OMT = ovomucin dissolved at pH 12.0 and hydrolyzed with 1% trypsin, OMPa = ovomucin dissolved at pH 12.0 and hydrolyzed with 1% papain, OMAl = ovomucin dissolved at pH 12.0 and hydrolyzed with 1% alcalase). [a-c]Values are mean with standard error. Values with different letters are significantly different ($P < 0.05$).

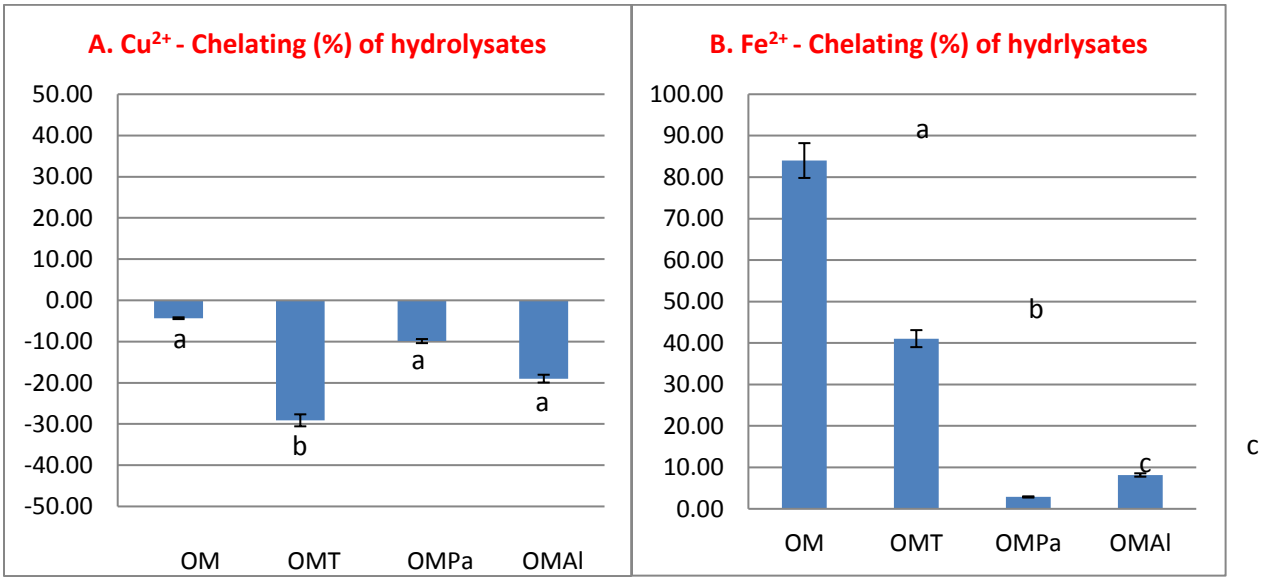

Table 1. Effects of enzyme treatments on the sizes and number of peptides produced from ovalbumin[1]

	Enzyme treatments			
MW of peptides (Da)	OM	OMAl	OMPa	OMT
700-1,000	4	0	7	61
1,000-1,500	24	0	93	214
1,500-2,000	33	0	64	184
2,000-3,000	33	0	9	184
3,000-5,000	55	0	0	100
Total (ProtScores)	149 (0.43)	0	207 (5.82)	743 (34.42)

[1]Peptides are identified using a LC-nano ESI-MS/MS. Peptides with ProtScores > 2.0, > 1.3, and > 0.47 have > 99%, > 95%, and > 66% probability, respectively.
Treatments: OM = ovomucin hydrolyzed with heating at 100 ºC for 15 min at pH 12.0, OMT = Ovomucin dissolved at pH 12.0 and hydrolyzed with 1% trypsin, OMPa = Ovomucin dissolved at pH 12.0 and hydrolyzed with 1% papain, OMAl = Ovomucin dissolved at pH 12.0 and hydrolyzed with 1% alcalase.