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Enzymatic hydrolysis of ovomucin and the functional and structural characteristics of peptides in the hydrolysates

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ABSTRACT

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 °C for 15 min 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 700–5000 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.

1. Introduction

Ovomucin is a sulfated glycoprotein with molecular weight ranges from 150 to 23,000 kDa and is responsible for the gel-like structure of thick in egg albumin (Tomimatsu & Donovan, 1972). There are two types of ovomucin: α- and β-types (Hiidenhovi, Aro, & Kankare, 1999; Li-Chan, Powrie, & Nakai, 1995). α-Ovomucin has long coiled regions and has more polymerized macromolecule than the β-type. Gel electrophoresis confirmed that α-ovomucin has two subunits called α1 and α2. On average, ovomucin has about 33% carbohydrates (Li-Chan & Kim, 2008), but β-type contains higher level of carbohydrates than α-type.

Although ovomucin was first separated in 1898, further studies have been a difficult task due to its insolubility in water unless denaturing agents like sodium dodecyl sulfate (SDS) and β-mercaptoethanol are present (Sleigh, Melrose, & Smith, 1973). Several methods, which include sonication, homogenization and the addition of chemicals like 6 M-guadinium, have been used to dissolve ovomucin in order to use in food and drug industries (Omana, Wang, & Wu, 2010; Robinson & Monsey, 1975; Sato, Hayakawa, & Nakamura, 1976). Ovomucin is widely used as a tumor suppressing agent in the body and can be used as an antiviral agent (Tanizaki, Tanaka, Iwata, & Kato, 1997). Ovomucin also has the capacity to reduce serum cholesterol level (Li-Chan & Kim, 2008).

Enzymatic hydrolysis is one of the methods to improve functional properties of ovomucin: Moreau, Nau, Piot, Guerin, and Brule (1997) found that flavourzyme solubilized ovomucin by hydrolyzing the protein. Hydrolysis of ovomucin with neutrase, flavourzyme, alcalase, or pronase E produced peptides with strong foaming capacities, but had low foam stabilities (Hammershøj, Nebel, & Carstens, 2008). Kobayashi et al. (2004) reported that the pronase hydrolysis of ovomucin produced an ovomucin glycopeptide that has *Escherichia 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. Others also reported that glycopeptides from ovomucin or subunits of ovomucin showed specific binding to Newcastle Disease Virus (Tsuge, Shimoyamada, & Watanabe, 1997), and had anti-tumor effects (Oguro, Ohaki, Asano, Ebina, & Watanabe, 2001; Watanabe, Tsuge, Shimoyamada, Ogama, & Ebina, 1998) or antiviral activities (Watanabe, Tsuge, & Shimoyamada, 1998).

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Chang et al. (2013) reported that the hydrolysates of ovomucin had strong antioxidant effects. The ovomucin hydrolysates reduced oxidation by 85% and >90% of the antioxidant activity was retained after 24 h, indicating that ovomucin hydrolysates can be used as an antioxidant in food processing. They also found that peptides with the amino acid sequences of LDEPDPL and NIQTDDFRT had strong antioxidant activities. The functional characteristics of peptides from other egg white proteins were also studied: peptides derived from ovalbumin, especially those with amino acid sequence of Phe-Arg-Ala-Asp-His-Pro-Phe-Leu (ovokinin) and Arg-Ala-Asp-His-Phe-Leu (ovokinin 2–7), showed very good ACE inhibitory activities (Miguel, Aleixandre, Ramos, & Fandiño, 2006). Peptides derived from ovotransferrin possessed an ability to control the growth of various cancer cells including lung (A549 and SK-MES-1), stomach (AGS), breast (MCF-7), larynx (Hep-2), cervix (HeLa) and liver (HepG2) cell lines (Moon et al., 2013). However, little information on the functional, structural and structural information of the peptides derived from ovomucin is available. 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.

2. Materials and methods

2.1. Materials

Medium-size, fresh brown chicken eggs (less than 5 days old) were purchased from a local market and used within 2 days. Standard enzymes, pepsin (2500–3500 U/mg protein, P6887), trypsin (~10,000 BAEE U/mg protein, T8003), Alcalase® 2.4L (protease from *Bacillus licheniformis*) (>2.4 U/g solution, P4860) and papain (10 U/mg protein, P4762) were purchased from Sigma–Aldrich (St. Louis, MO, USA), and other chemicals were purchased from Fisher Scientific (Thermo Fisher Scientific Inc., Waltham, MA, USA). Ovomucin was isolated from hen eggs according to the method of Abeyrathne, Lee, and Ahn (2013).

2.2. Methods

2.2.1. Hydrolysis of ovomucin

The lyophilized ovomucin (Abeyrathne et al., 2013) 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 °C for 0, 3, 6, 9, 12 and 24 h. The enzyme: substrate ratio was on the basis of the original form of enzymes purchased from Sigma. At the end of incubation, the samples were heated at 100 °C for 15 min to inactivate the enzyme. Also, hydrolysis of ovomucin by heating (100 °C for 15 min) under alkaline conditions (pH 12.0) was tested.

2.2.2. Sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE)

SDS–PAGE gels (15%) were prepared to analyze the peptides derived from ovomucin. SDS–PAGE was conducted under reducing conditions using a Mini-Protein II cell (Bio-Rad) and Coomassie Brilliant Blue R-250 (Sigma) was used to stain the gels (Price & Nairn, 2009).

2.2.3. Measurement of functional properties

Ovomucin was hydrolyzed using trypsin (OMT), papain (OMPa), or alcalase (OMAl) for 3 h at 37 °C as described above (Section 2.2.1). At the end of the hydrolysis, the enzymes were heat-inactivated at 100 °C for 15 min. Ovomucin was also treated with heat at (100 °C for 15 min) under alkaline conditions (pH 12.0) and used as OM treatment. The degree of hydrolyzing was analyzed using SDS–PAGE pictures and physical appearance (turbidity or precipitation) of the hydrolyzed products.

2.2.3.1. Antioxidant activity. An oil-in-water emulsion was prepared by homogenizing 1.0 g of corn oil (Ottogi Company, Seoul, Korea) and 100 μl of tween-20 with 100 ml of distilled water using a polytron homogenizer (Kinematica, Switzerland) for 2 min in an ice bath at full power. Samples for lipid oxidation assay was prepared by mixing 8 ml of oil emulsion, 0.5 ml of 0.2% ascorbic acid (Sigma–Aldrich, USA), 0.5 ml of 200 ppm Fe^{2+} ($FeSO_4$, Sigma) and 1 ml of hydrolyzed products of ovomucin. The samples were incubated at 37 °C for 16 h. At the end of incubation, one ml of sample was transferred and added with 2 ml of thiobarbituric acid/trichloroacetic acid solution (20 mM TBA/15% TCA) and 50 μl of 10% butylated hydroxyanisole in 90% ethanol, and then vortex-mixed. The mixture was incubated in a 90 °C water bath for 15 min to develop color. The sample was cooled in an ice bath for 10 min and centrifuged at 3000×*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 TBARS were expressed as mg of malondialdehyde (MDA) per L of emulsion.

2.2.3.2. ACE-inhibitory activity. ACE-inhibitory activity of the hydrolysates was measured using the methods of Miguel, Alonso, Salaices, Aleixandre, and Fandiño (2007) and Yu et al. (2010) with some modifications. An aliquot of hydrolyzed sample (40 μl) was mixed with 100 μl of 0.1 M borate buffer (pH 8.3) containing 5 mM of HHL (N-Hippuryl-L-Histidil-L-Leucine), 0.3 M NaCl and 20 μl of ACE (0.1 U). The mixture was incubated at 37 °C for 30 min, and the reaction was stopped by adding 150 μl of 1 M HCl. The hippuric acid formed was extracted with 1000 μl of ethyl acetate and centrifuged at 1500×*g* for 10 min. The organic phase (750 μl) was transferred to a culture tube (13 × 100 mm) and dried in a heat block under nitrogen gas stream at 95 °C for 10 min. The dried sample was dissolved with 800 μl of distilled water and vortex mixed, and the absorbance was measured at 228 nm against a blank prepared with 40 μl of distilled water instead of sample. Three replicates were carried out for each treatment. ACE-inhibitory activity was measured using the following equation.

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

2.2.3.3. Fe^{2+}-chelating activity. The Fe-chelating activity of the hydrolysates was measured using the Ferrozine method (Carter, 1971) with some modifications. One hundred microliter of the hydrolysates of ovomucin was mixed with 1 ml of distilled water and 1 ml of 10 ppm Fe^{2+} ($FeSO_4$, Sigma) in a 15-ml Falcon tube, and incubated for 5 min at room temperature. To remove the proteins and peptides in the sample, 900 μl of 11.3% trichloroacetic acid (TCA) was added and then centrifuged at 2500×*g* for 10 min. One milliliter of the supernatant was transferred to a disposable culture tube and 1 ml of distilled water, 800 μl of 10% ammonium acetate (Fisher Scientific) and 200 μl 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 vortex mixed. After