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Effect of proteolysis on the sialic acid content and bifidogenic activity of ovomucin hydrolysates

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ABSTRACT

Ovomucin, accounting for ~3.5% of egg white proteins, contains 2.6–7.4% of sialic acid; sialic acid is suggested to play important roles in host-recognition, cognition and memory development. However, ovomucin's limited water solubility might restrict its future applications. The objective of the study was to examine the effect of proteolysis of ovomucin on the sialic acid content and bifidogenic activity of ovomucin hydrolysates. Ovomucin extract was hydrolyzed by 14 proteases with yields and DHs ranging from 42.6% (flavourzyme) to 97.4% (protease N), and 2.4% (flavourzyme) to 46.3% (pronase), respectively. Ovomucin hydrolyzed by pronase and protex 26L showed molecular weight (M_w) distributions less than 40 kDa while others larger than 200 kDa. Allergenicity of ovomucin hydrolysates was significantly reduced ($P < 0.05$) in comparison to ovomucin extract. The content of sialic acid in hydrolysates ranged from 0.1% (protex 26L) to 3.7% (pronase). Ovomucin hydrolysates did not generally support growth of *Bifidobacterium* spp. *in vitro*.

1. Introduction

Egg white is widely used in the food industry due to its excellent gelling, foaming and emulsifying properties (Wang & Wang, 2009). Ovomucin, a glycoprotein accounting for 2–4% of total egg white protein, is a major contributor to egg white functionality (Kato, Oda, Yamanaka, Matsudomi, & Kobayashi, 1985). It is composed of a carbohydrate-poor and a carbohydrate-rich subunit, containing 11–15% and 50–57% (w/w) carbohydrate, respectively (Robinson & Monsey, 1971; Wang & Wu, 2012). Based on its solubility, ovomucin can be classified into an insoluble form and a soluble form with molecular weights of 23,000 kDa and 8300 kDa, respectively (Tominatsu & Donovan, 1972). Generally, ovomucin is highly insoluble at neutral pH or in the absence of denaturing agents.

Protein hydrolysis is widely applied to improve protein functionalities including solubility and bioactivities. The solubility of ovomucin increases at increasing degrees of hydrolysis (DH); ovomucin hydrolysates show relatively high foaming ability at DH of 15–40% (Hammershøj, Nebel, & Carstens, 2008; Hiidenhovi, Hietanen, Mäkinen, Huopalahti, & Ryhänen, 2005). Glycopeptides prepared from pronase digestion of ovomucin, show anti-adhesive activity against *Escherichia coli* O157:H7 (Kobayashi et al., 2004), and anti-tumor activity in a double grafted tumor system (Oguro, Watanabe, Tani, Ohishi, & Ebina, 2000). In addition, two peptides (LDEPDPL and NIQTDDFRT) with radical scavenging activity have been identified from ovomucin hydrolysate (Chang et al., 2013).

Ovomucin is rich in sialic acid (2.6–7.4%, w/w), which plays important roles in various biological processes and in infant nutrition (Robinson & Monsey, 1971; Tang, Liang, Cai, & Mou, 2008). Sialic acid acts as recognition sites for microorganisms, toxins and hormones, protects cells from enzymatic hydrolysis and immunological attacks, and is involved in intermolecular and intercellular interactions (Spichtig, Michaud, & Austin, 2010). As an important component of brain gangliosides and polysialylated neural cell adhesion molecules, sialic acid is thought to play crucial roles in cognition and memory development in infants. Sialic acid has been suggested as an essential nutrient for infants (Wang & Brand-Miller, 2003). The sialic acid in ovomucin is *N*-Acetylneuraminic acid, which is identical to the sialic acid found in human glycans but different from the *N*-Glycolylneuraminic acid present in glycans of other mammals. Ovomucin may thus serve as source of sialic acid in human nutrition (Schauer, Srinivasan, Coddeville, Zanetta, & Guérardel, 2009). However, the effect of hydrolysis on the sialic acid content in the hydrolysates has not been studied. Furthermore, sialic acid-containing substances were suggested to promote the growth of bifidobacteria

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(Idota, Kawakami, & Nakajima, 1994), indicating the bifidogenic potential of ovomucin hydrolysate. Interestingly, porcine gastric mucin, a member of mucin family, was reported to have bifidogenic activity (Killer & Marounek, 2011). Thus, it is interesting to explore whether ovomucin hydrolysates support the growth of bifidobacteria.

The objectives of the study were to determine the effect of proteolysis of ovomucin on the sialic acid content and bifidogenic activity of ovomucin hydrolysates. Ovomucin hydrolysates were prepared by proteolysis and the hydrolysis yield, nitrogen recovery, amino acid composition, peptide profile, M_w distribution, degree of hydrolysis, sialic acid contents, and the growth of bifidobacteria with ovomucin as the sole carbohydrate source were studied. Since egg is a major allergen, the effect of hydrolysis on allergenicity of ovomucin extracts was also determined.

2. Materials and methods

2.1. Materials and chemicals

Fresh eggs from White Leghorn were obtained within 24 h from the Poultry Research Centre of the University of Alberta (Edmonton, Canada) and used on the same day for extraction of ovomucin. 2,4,6-Trinitrobenzenesulfonic acid solution (TNBS), sodium dodecyl sulfate (SDS) and trifluoroacetic acid (TFA) were all obtained from Sigma-Aldrich (St. Louis, MO, USA). Tween 20 and Coomassie brilliant blue (CBB) R-250 were obtained from Bio-Rad (Bio-Rad Laboratories, Inc., Hercules, CA). Hydrochloric acid and sodium hydroxide were bought from Fisher Scientific Inc. (Fisher Scientific, Ottawa, ON, Canada). Standard proteins (ovalbumin, ovotransferrin, ovomucoid, and lysozyme) were provided by Neova Technologies Inc. (Abbotsford, BC, Canada). Milli-Q water was prepared by the Milli-Q water supply system (Millipore Corporation, Billerica, MA, USA).

2.2. Extraction of ovomucin from egg white

Ovomucin was extracted as we previously reported (Wang, Omana, & Wu, 2012). In brief, fresh egg white was diluted with 3 times of Milli-Q water, and stirred for 120 min. After adjusting the pH to 5.0 by 2 N HCl, the slurry was placed in cold room (4 °C) for 24 h and centrifuged at 15,344*g* for 10 min at 4 °C (Beckman Coulter, Rotor JA10, USA). The precipitate was collected, lyophilized and stored at −20 °C until further analysis.

2.3. Hydrolysis of ovomucin by different enzymes

To prepare the hydrolysates, ovomucin extract was dispersed into Milli-Q water to make a 1% (w/v, ovomucin/water) slurry. The pH and temperature of the slurry was adjusted to appropriate conditions of the individual enzymes (Table 1) and then enzyme was added at a level of 2% (w/w, enzyme/substrate). Hydrolysis was performed on a Titrando (842, Metrohm, Herisan, Switzerland) equipped with a circulating water bath during hydrolysis to maintain a constant pH and temperature for 3 h for pepsin and pancreatin or 4 h for the other 12 enzymes. After incubation, the suspension was heated at 95 °C for 15 min in a water bath, cooled to ambient temperature on ice and then centrifuged at 15,344*g* for 20 min at 4 °C to remove the precipitate (Beckman Coulter, Rotor JA14, USA). The supernatant was collected and lyophilized. All hydrolysis were conducted in duplicate. The hydrolysis yield and the nitrogen recovery (NR) were calculated as follows:

$$\text{Hydrolysis yield}\,(\%) = \frac{\text{Sample weight in supernatant}}{\text{Sample weight before hydrolysis}} \times 100$$

$$\text{Nitrogen recovery}\,(\%) = \frac{\text{Nitrogen content in supernatant}}{\text{Nitrogen content in original substrate}} \times 100$$

2.4. Characterization of ovomucin and ovomucin hydrolysates

2.4.1. The purity of the prepared ovomucin

The purity of the ovomucin extract was determined by a High-load 16/60 column (Superdex 200 preparatory grade) coupled with Fast Performance Liquid Chromatography (FPLC) (GE Healthcare Bio-Sciences AB, Uppsala, Sweden) as previously reported (Omana & Wu, 2009). The prepared ovomucins were dissolved in 100 mM sodium phosphate buffer (pH 7.0) containing SDS (50 g/l) and β-mecaptoethanol (10 ml/l) at a concentration of 5 g/l. Samples were filtered through a 0.45 μm filter (PVDF, Mandel Scientific Company Inc.) prior to FPLC analysis. The injection volume was 3 ml, and the column was eluted with 100 mM phosphate buffer (pH7.0) containing 5 g/l of SDS and 1 ml/l of β-mecaptoethanol at a flow rate of 1 ml/min. The eluate was monitored by a UV detector at 280 nm. The percentages of other proteins (ovalbumin, ovomucoid, and lysozymein) in ovomucin extracts were calculated from their respective standard curves, and the purity of ovomucin was calculated by subtracting the

Table 1
Enzymatic conditions and characterization of ovomucin hydrolysates.

Enzymes	pH	Temp. (°C)	Origin	Supplier	Protein content (w/w, %)‡	Yield (%)‡	Nitrogen recovery (%)‡	Sialic acid content (w/w, %)‡	Degree of hydrolysis (%)‡
Protease N	7.5	55	*Bacillus subtilis*	Amano Pharmaceutical Co.	81.8 ± 0.4	97.4 ± 0.6	93.8 ± 0.4	2.1	8.5 ± 1.5
Protex 51FP	7.5	50	*Aspergillus oryzae*	Genencor Division of Danisco	82.9 ± 1.5	66.3 ± 0.3	64.7 ± 1.2	1.7 ± 0.1	3.6 ± 1.8
Protex 6L	9.5	60	*Bacillus licheniformis*	Genencor Division of Danisco	83.1 ± 0.1	54.6 ± 3.4	53.5 ± 0.1	0.8 ± 0.2	3.1 ± 0.8
Trypsin VI	8.0	37	Porcine pancreas glands	Neova Technologies Inc.	85.6 ± 0.4	84.4 ± 1.7	85.1 ± 0.4	2.0 ± 0.3	3.8 ± 0.7
Protex 26L	3.0	50	*Aspergillus niger*	Genencor Division of Danisco	83.4 ± 0.2	70.8 ± 2.1	69.5 ± 0.2	0.1	11.8 ± 3.5
Protease P	7.0	45	*Aspergillus melleus*	Amano Pharmaceutical Co.	80.1 ± 0.2	88.5 ± 1.3	83.5 ± 0.2	2.4 ± 0.1	24.1 ± 1.3
Alcalase 2.4L	8.0	50	*Bacillus licheniformis*	Sigma Chemical Co.	81.8 ± 0.3	75.8 ± 0.9	73.1 ± 0.3	2.1	4.0 ± 0.3
Protease M	5.0	45	*Aspergillus oryzae*	Amano Pharmaceutical Co.	80.8 ± 0.4	49.0 ± 1.0	46.6 ± 0.2	1.7 ± 0.1	17.4 ± 0.5
Pepsin + Pancreatin	2.0/6.5	37	Porcine pancreas and gastric	Sigma Chemical Co.	69.0 ± 0.7	83.4 ± 1.7	67.8 ± 0.7*	1.7 ± 0.2	17.6 ± 3.7
Acid protease II	3.5	45	*Rhizopus niveus*	Amano Pharmaceutical Co.	84.6 ± 1.0	58.1 ± 1.2	57.9 ± 0.7	0.3	7.4 ± 1.1
Pepsin	2.0	37	Porcine gastric	Sigma Chemical Co.	85.9 ± 1.7	81.0 ± 1.5	81.9 ± 1.6	0.8 ± 0.1	6.1 ± 3.4
Flavourzyme	7.0	50	*Aspergillus oryzae*	Sigma Chemical Co.	82.9 ± 2.0	42.6 ± 0.6	41.6 ± 1.0	0.5 ± 0.2	2.4 ± 1.0
Protease A	7.5	50	*Aspergillus oryzae*	Amano Pharmaceutical Co.	84.1 ± 0.1	59.1 ± 0.7	58.5 ± 0.1	1.3 ± 0.1	3.8 ± 0.6
Pronase	7.5	50	*Streptomyces griseus*	Roche Diagnostics GMBH	80.0	91.1 ± 0.3	85.8 ± 0.1*	3.7	46.3 ± 2.2

‡ Data were expressed as Mean ± SD.
* Nitrogen recovery was significantly different from the corresponding yield ($P < 0.05$).