



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*.

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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 & Marounnek, 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 15,344g 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 Titrand (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 15,344g 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 lysozyme) 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	<i>Bacillus subtilis</i>	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	<i>Aspergillus oryzae</i>	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	<i>Bacillus licheniformis</i>	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	<i>Aspergillus niger</i>	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	<i>Aspergillus melleus</i>	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	<i>Bacillus licheniformis</i>	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	<i>Aspergillus oryzae</i>	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	<i>Rhizopus niveus</i>	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	<i>Aspergillus oryzae</i>	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	<i>Aspergillus oryzae</i>	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	<i>Streptomyces griseus</i>	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 ($P < 0.05$).

amount of other proteins (Hiidenhovi, Mäkinen, Huopalahti, & Ryhänen, 2002; Omana & Wu, 2009).

2.4.2. Degree of hydrolysis (DH) and sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE)

DH was measured according to the TNBS method (Adler-Nissen, 1979). SDS-PAGE was carried out by 7.5% ready-to-use gels (Bio-Rad Laboratories, Inc., Hercules, CA). Protein and carbohydrate bands in the gel were stained with CBB R-250 and periodic acid-Schiff (PAS) reagents (Glycoprotein Detection Kit, Sigma Chemical Co.), respectively (Kobayashi et al., 2004).

2.4.3. Quantification of sialic acid in ovomucin hydrolysate

Samples were acid hydrolyzed by 0.1 M HCl at a concentration of 2 g/l, at 80 °C for 1 h (Rohrer, Thayer, Weitzhandler, & Avdalovic, 1998). The amount of sialic acid in ovomucin hydrolysates was determined by high-performance anion-exchange chromatography with pulsed amperometric detection (HPAEC/PAD) using a Dionex ICS-3000 Ion Chromatography system on a Carbowac PA20 column (3 × 150 mm, Dionex, Oakville, Canada). *N*-Acetylneuraminic acid was used as standard (Rohrer et al., 1998). The injection volume was 10 µl and the column temperature was kept at 30 °C. The column was eluted using a gradient, composed of 100 mM NaOH (A) and 1 M sodium acetate in 100 mM NaOH (B), 7–30% B from 0 to 7.5 min, 30% B from 7.5 to 9 min, 30–7% B from 9.0 to 9.5 min, and 7% B from 9.5 to 16.5 min, at a flow rate of 0.35 ml/min.

2.4.4. Amino acid composition

Amino acid composition was analyzed by an HPLC method on a Supelcosil 3 µm LC-18 reverse phase column (4.6 × 150 mm; Sigma-Aldrich, St. Louis, MO, USA) equipped with a guard column (4.6 × 50 mm; Sigma-Aldrich, St. Louis, MO, USA) packed with Supelco LC-18 reverse phase packing (2040 µm) using a Fluorichrom detector (excitation 340 nm emission 450 nm). Samples were hydrolyzed under a nitrogen atmosphere in 6 N HCl at 110 °C for 24 h to determine all the amino acids except cysteine, methionine, proline, and tryptophan (Jarrett, Cooksy, Ellis, & Anderson, 1986). Methionine and cysteine were determined by oxidation using performic acid hydrolysis (Moore, 1963) and tryptophan was released by alkaline hydrolysis before qualification (Ravindran & Bryden, 2005). Amino acid composition was expressed as g of amino acid/100 g of hydrolysate.

2.4.5. Size exclusion chromatography

Molecular weight (M_w) distributions of ovomucin hydrolysate were analyzed by a TSK-Gel G3000SWxl gel filtration column (7.8 mm × 30 cm, 7 µm, Tosoh Bioscience LLC, Montgomeryville, PA, USA) coupled with a guard column (6.0 mm × 4.0 cm, 7 µm, Tosoh Bioscience LLC, Montgomeryville, PA, USA) attached to a Waters 600 HPLC system (Waters Corporation, Milford, MA, USA). Samples, at a concentration of 10 g/l, were prepared by dissolving hydrolysates in 100 mM sodium phosphate buffer (pH 6.7) containing 0.05% (w/v) of sodium azide and 100 mM of sodium sulfate. The samples were automatically injected by a Waters 2707 autosampler at a volume of 25 µl, and the column was eluted isocratically with 100 mM sodium phosphate buffer (pH 6.7) containing 0.05% (w/v) of sodium azide and 100 mM of sodium sulfate at a flow rate of 0.5 ml/min for 40 min, and monitored by a Waters 2998 photodiode array at 280 nm (Wang et al., 2012).

2.4.6. Peptide profiles of ovomucin hydrolysates

Hydrolysates were dissolved in water (HPLC grade) containing 0.1% trifluoroacetic acid (TFA), and separated on an Acquity Ultra-Performance Liquid Chromatograph (UPLC) system with an Acquity UPLC BEH C₁₈ column (100 mm × 2.1 mm i.d., 1.7 µm,

Waters, Milford, MA, USA) using a linear gradient of 100–60% of solvent A (water in 0.1% TFA) within 30 min; while solvent B was acetonitrile in 0.1% TFA. The sample concentration was 10 mg/ml and injection volume was 10 µl. Separation was performed at 40 °C at a flow rate of 0.2 ml/min. The elution was detected at 220 nm.

2.5. Enzyme-linked immunosorbent assay (ELISA)

Allergenicity of ovomucin hydrolysate was evaluated using a half-sandwich ELISA format (Li, Offengenden, Fentabil, Gänzle, & Wu, 2013), to determine the IgE binding activity. Freeze dried ovomucin hydrolysates were dissolved in 50 mM sodium carbonate buffer (pH 9.6) at a concentration of 5 g/l, then 100 µl of the sample was applied onto each well of the 96-well flat bottomed microtiter plates (Costar, Corning, Inc, NY) and incubated at 4 °C overnight. The plate was washed 4 times with phosphate buffered saline containing 0.05% Tween 20 (PBST, pH 7.2, 200 µl/well), then blocked with 150 µl/well of 2% bovine albumin serum (BSA, Sigma Chemical Co.) in 50 mM sodium carbonate buffer at pH 9.6 for 2 h at 37 °C with gentle shaking. The plate was washed again with PBST three times. Human plasma from patients with egg-white allergy (PlasmaLab International, Everett, WA, USA) were diluted 1:25 in PBST containing 1% BSA, then 100 µl/well was applied to the plate and incubated overnight at room temperature with gentle shaking. After washing, the plate was incubated overnight with 100 µl/well of goat anti-human IgE secondary antibody (Sigma Chemical Co.) at ambient temperature with gentle shaking, which was diluted 1:2000 in PBST containing 1% BSA. Finally, the plate was washed again with PBST before the addition of 100 µl/well of p-nitrophenyl phosphate (pNPP, Sigma Chemical Co.) for developing color at room temperature while shaking. After 45 min, the reaction was stopped by adding 25 µl/well of 3 M sodium hydroxide and absorbance was read at 405 nm. The acidified egg white was used as positive control (Kovacs-Nolan, Zhang, Hayakawa, & Mine, 2000) and all the final results were expressed after subtraction of blank value (<0.10).

2.6. Metabolism of ovomucin hydrolysates by bifidobacteria

2.6.1. Bacterial strains and growth conditions

The reference bacterial strains used in the study were *Bifidobacterium adolescentis* (American Type Culture Collection, ATCC 15703), *Bifidobacterium longum* (ATCC 15707), *Bifidobacterium bifidum* (ATCC 15696, ATCC 29521 and ATCC 35914), and *Bifidobacterium infantis* (ATCC 15697). The bacteria were fermented in modified MRS medium supplied with selected ovomucin hydrolysates as the sole carbohydrate source at 37 °C for 48 h under anaerobic conditions (10% of CO₂, 5% of H₂, and 85% N₂) (Schwab & Gänzle, 2011). The growth was measured as turbidity at 600 nm at interval of 0, 8, 24, and 48 h.

2.6.2. Analysis of metabolite and degradation of carbohydrates in ovomucin hydrolysates

The concentration of lactate was analyzed at fermentation intervals of 0, 8, 24, and 48 h by HPLC with a refractive index detector and an Aminex HPX-87 column (300 mm × 7.8 mm, Bio-Rad) at 70 °C. 5 mM of H₂SO₄ was used as the solvent and the flow rate was 0.4 ml/min. For sample preparation, equal volumes of 7.5% perchloric acid were added to the supernatants of fermentation liquor to precipitate the proteins after overnight incubation at 4 °C. The precipitates were removed by centrifugation at 8603g for 5 min at room temperature (Galle, Schwab, Arendt, & Gänzle, 2010).

After fermentation, carbohydrates in ovomucin medium were hydrolyzed by incubating for 2 h at 95 °C in 2 M HCl. Resulting monosaccharides were determined on a Dionex ICS-300 system

equipped with a CarbowacPA20 column (Dionex, Oakville, Canada) using water (A) and 200 mM NaOH (B) as eluents at a flow rate of 0.25 ml/min with the following gradient: 0 min 6% B and 20 min 100% B. Mannose, galactose, galactosamine hydrochloride, and glucosamine hydrochloride (all purchased from Sigma Chemical Co.), which are present in ovomucin, were applied as external standards (Galle et al., 2010; Schwab & Gänzle, 2011).

2.7. Statistical analysis

All experiments were performed at least in triplicate and the results were expressed as mean $\pm$ standard deviation (SD). All statistical analysis was carried out using IBM SPSS statistics 19 (SPSS Inc, USA). Significant differences were defined at a 5% level ($P < 0.05$).

3. Results and discussion

3.1. Preparation of ovomucin hydrolysates

This study employed the one-step method for preparation of ovomucin (Wang et al., 2012). The purity of ovomucin extract was 55.7%, which was lower than the sample prepared by a

2-step method (Omana & Wu, 2009) but comparable to the purity of ovomucin preparations reported elsewhere (Hiidenhovi et al., 2002). The contents of ovalbumin, ovomucoid, and lysozyme were $20.0 \pm 1.5\%$, $3.3 \pm 0.1\%$, and $20.9 \pm 4.4\%$, respectively.

Previous studies on the hydrolysis of ovomucin were usually performed over a 24 h period with yields ranging from 67% to 98% (Hammershøj et al., 2008; Kobayashi et al., 2004; Oguro et al., 2000). Our preliminary study showed 4 h of digestion reached the plateau of ovomucin hydrolysis. The peptide yield prepared using our method ranged from 42.6% (flavourzyme) to 97.4% (protease N) and the nitrogen recovery (NR) ranged from 41.6% (flavourzyme) to 93.8% (protease N) (Table 1); these results compare favorably to previous results. Most ovomucin hydrolysates exhibited similar NR and yield values except for pepsin-pancreatin and pronase hydrolysates. The NR of the pepsin-pancreatin hydrolysate and pronase hydrolysate (67.8% and 85.8%, respectively) was significantly lower ($P < 0.05$) than their yields (83.4% and 91.1%, respectively), indicating the presence of a higher proportion of carbohydrate moieties in these hydrolysates.

Degree of hydrolysis (DH) ranged from 2.4% to 46.3% (Table 1). DH showed a positive correlation with the yield of hydrolysates. However, Protease N showed the highest yield (97.4%) but with a

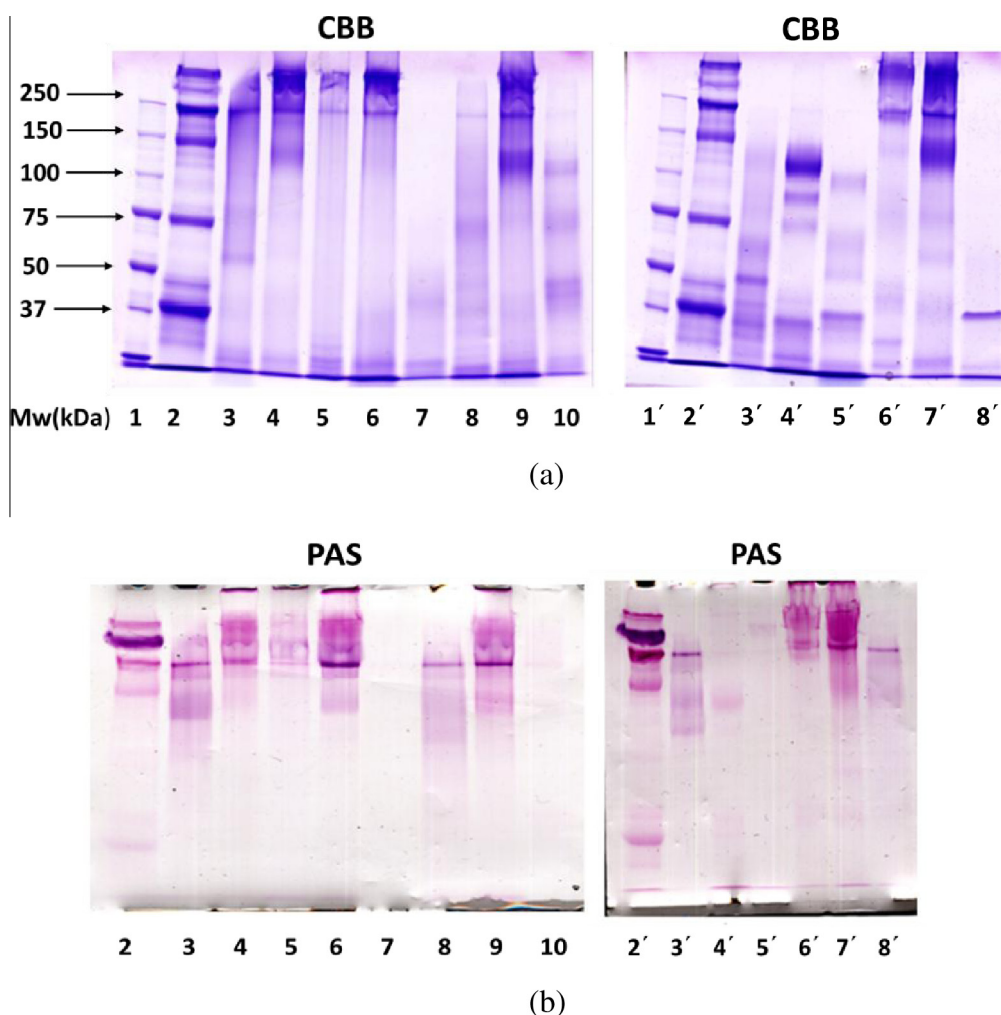


Fig. 1. SDS-PAGE analysis of ovomucin hydrolysates. The gels were stained with CBB (a) and Schiff reagents (b). Lanes 1–10 were molecular weight (M_w) marker (1), ovomucin reduced by β -mercaptoethanol (2), ovomucin digested by protease N (Ovomucin-Protease N) (3), Ovomucin-Protex 51FP (4), Ovomucin-Protex 6L (5), Ovomucin-Trypsin VI (6), Ovomucin-Protex 26L (7), Ovomucin-Protease P (8), Ovomucin-Alcalase 2.4L (9), and Ovomucin-Protease M (10). Lanes 1'–8' were M_w marker (1), Ovomucin reduced by β -mercaptoethanol (2), Ovomucin-pepsin and pancreatin (3), Ovomucin-Acid protease II (4), Ovomucin-Pepsin (5), Ovomucin-Flavourzyme (6), Ovomucin-Protease A (7), and Ovomucin-Pronase (8).

relatively low DH (8.5%). A high yield associated with a low DH was probably due to a lack of cleavage sites in the substrate for Protease N although it could partially hydrolyze ovomucin and improve its solubility (Hiidenhovi et al., 2005).

3.2. Molecular weight distribution of ovomucin hydrolysates

Different enzymes displayed different protease activities against ovomucin. As shown in Fig. 1(a), major bands over 150 kDa in ovomucin extracts were noticeably degraded by protex 26L, protease M, pepsin/pancreatin, acid protease II, pepsin, protease P, and pronase. Protease 26L and pronase were the two most efficient enzymes, as the dominant polypeptides released by these two enzymes were all below 40 kDa (Fig. 1a). PAS staining showed the presence of glycopeptides bands larger than 100 kDa in most hydrolysates with the exception of protease 26L and protease M hydrolysates; although acid protease II and pepsin hydrolysates gave relatively light bands (Fig. 1(b)).

The M_w distribution of ovomucin hydrolysates were further analyzed by size exclusion chromatography (Fig. 2(a)). Hydrolysates prepared by protease 6L, flavourzyme, trypsin VI, alcalase 2.4L, protease A, protex 51FP were composed mainly of protein fragments larger than 200 kDa, indicating a low degree of degradation of ovomucin. On the other hand, hydrolysates prepared by pepsin followed by pancreatin, protease P, protease M, protex 26L and pronase are composed of protein fragments with M_w less

than 12.4 kDa. In general, a higher DH resulted in hydrolysates with a smaller M_w .

3.3. Sialic acid amounts of ovomucin hydrolysates

The ovomucin extract contains 3.2% of sialic acid, which was in the range of the reported data (2.6–7.4%, w/w) (Feeney, Rhodes, & Andersos, 1980; Sleight, Melrose, & Smith, 1973). Sialic acid contents in ovomucin hydrolysates ranged from 0.1% to 3.7% (Table 1). Hydrolysates prepared by pronase, protease N, protease P, trypsin VI, and alcalase 2.4L showed relatively high sialic acid contents, whereas those of protex 26L and acid protease II were extremely low (0.1% and 0.3%, respectively). The extreme low level of sialic acid in these two hydrolysates might be due to the applied acidic pH (pH 3.0) during digestion and high temperature (95 °C) during deactivating the enzyme, which were known to release or degrade sialic acid (Spichtig et al., 2010).

3.4. Amino acid composition

Amino acid composition of ovomucin and ovomucin hydrolysates are listed in Table 2. All ovomucine hydrolysates contained similar amounts (g/100 g of ovomucin) of essential amino acids except the hydrolysate prepared by pepsin and pancreatin. Pepsin-pancreatin hydrolysates had relatively lower amounts of essential amino acids probably due to its lower content of protein (Table 1). Ovomucin extracts in the present study contained more essential amino acids except His compared to the published data as shown in Table 2 (Robinson & Monsey, 1971). After hydrolysis, the amino acid composition of ovomucin hydrolysates did not change except for Phe and Cys. The amount of essential amino acids in all the hydrolysates meets the FAO/WHO/UNU reference pattern for adults, and some of them were also higher than the model value

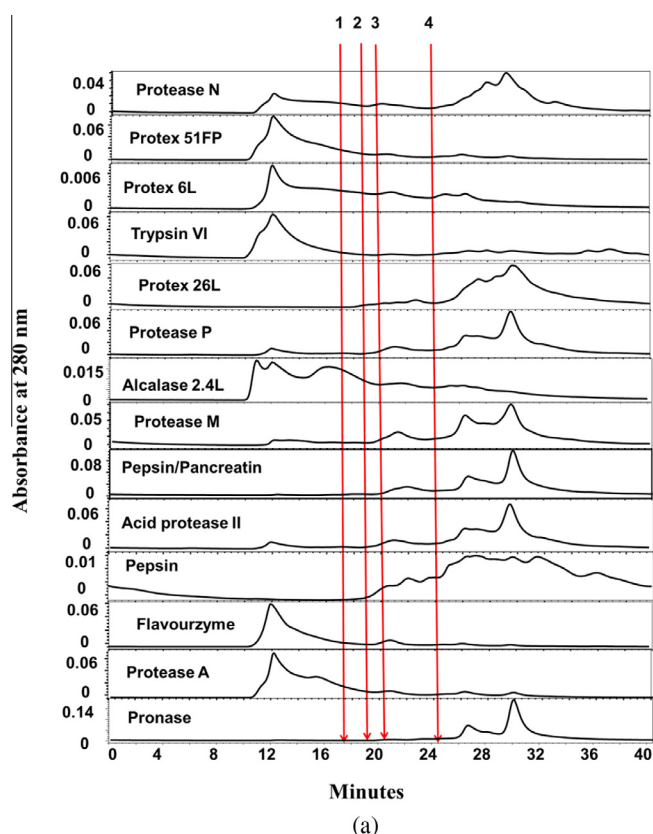


Fig. 2. (a) Gel filtration chromatograms of ovomucin hydrolysates. The sample was eluted isocratically with 100 mM sodium phosphate buffer (pH 6.7) containing 0.05% (w/v) of sodium azide and 100 mM of sodium sulfate at a flow rate of 0.5 ml/min for 40 min. The cut-off value 1–4 were protein standards with molecular weights of 200, 150, 66, and 12.4 kDa, respectively. The proteases in the picture represented the corresponding ovomucin hydrolysates. (b) HPLC elution profiles of ovomucin hydrolysates on an Acquity UPLC BEH C18 column. HPLC was carried out with a linear gradient of 100–60% of solvent A (water in 0.1% TFA) within 30 min at 0.2 ml/min at 220 nm; solvent B was acetonitrile in 0.1% TFA. The proteases in the picture represented the corresponding ovomucin hydrolysates.

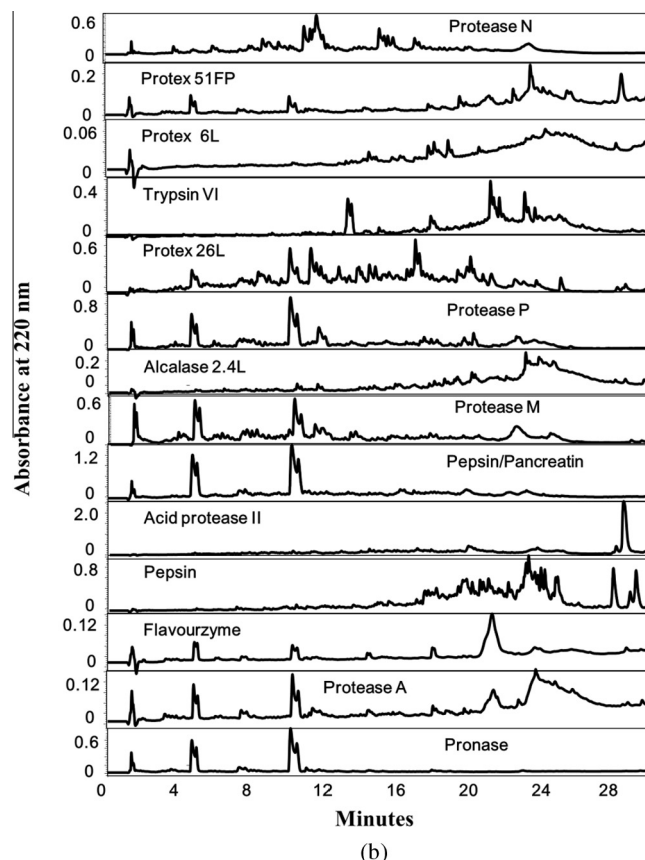


Fig. 2 (continued)

Table 2
Amino acid profiles of ovomucin (OvoM) and ovomucin hydrolysates (g/100 g of sample).

	OvoM- Protease N	OvoM- Protex 51FP	OvoM- Protex 6L	OvoM- Trypsin VI	OvoM- Protex 26L	OvoM- Protease P	OvoM- Alcalase 2.4L	OvoM- Protease M	OvoM- Pepsin & Pancreatin	OvoM-Acid Prottease II	OvoM- Pepsin	OvoM- Flavourzyme	OvoM- Protease A	OvoM- Pronase	OvoM	OvoM (3)	FAO/WHO/ UNO ^b (g/ 100 g of protein)	
																	Infant	Adult
<i>Essential amino acids</i>																		
Leu	5.0	5.0 ± 0.3	5.5 ± 0.7	6.0	5.0	5.0	5.5 ± 0.7	5.0	4.0	6.0	5.6 ± 0.2	6.0	5.5 ± 0.2	5.6 ± 0.8	5.3 ± 0.6	4.0	6.6	1.9
Ile	4.0	4.0 ± 0.3	4.0	4.0	4.0	4.0	4.0	4.0	3.0	4.5 ± 0.7	4.3 ± 0.1	5.0	4.1 ± 0.3	4.5 ± 0.9	4.1 ± 0.3	2.6	2.8	1.3
Lys	5.0	5.1 ± 0.4	5.0	5.5 ± 0.7	5.0	5.0	5.0	5.0 ± 1.4	4.0	5.5 ± 0.7	4.9 ± 0.3	6.0	5.4 ± 0.1	5.2 ± 0.4	5.3 ± 0.8	4.3	5.8	1.6
Met	2.5 ± 0.1	2.6 ± 0.3	2.0 ± 0.8	2.7 ± 0.1	1.3 ± 0.8	2.0 ± 0.8	2.1 ± 0.8	1.8 ± 0.1	1.7 ± 1.0	1.9 ± 0.4	1.6 ± 0.5	1.9 ± 0.6	1.3 ± 0.1	1.0 ± 0.4	2.4 ± 0.8	1.4		
(Met + Cys)	18.0 ± 0.9	19.4 ± 1.6	16.3 ± 5.0	16.5 ± 0.1	15.4 ± 0.3	17.0 ± 0.9	15.8 ± 0.4	14.7 ± 0.3	16.7 ± 0.3	17.0 ± 0.1	15.9 ± 4.0	15.6 ± 1.5	14.6 ± 0.1	11.3 ± 2.1	20.3 ± 3.5	5.5	2.5 ^c	1.7 ^c
Phe	4.0	4.2 ± 0.3	4.0	4.0	4.0	4.0	4.0	4.0	3.0	4.0	4.2 ± 0.2	5.0	4.5 ± 0.1	4.2 ± 0.5	3.9 ± 0.3	3.1		
(Phe + Tyr)	6.5 ± 0.7	7.1 ± 0.6	7.0	6.0	6.5 ± 0.7	6.5 ± 0.7	6.5 ± 0.7	6.0	5.5 ± 0.7	7.5 ± 0.7	6.8 ± 0.3	8.0	6.9 ± 0.1	6.4 ± 0.7	6.3 ± 0.4	5.6	6.3 ^d	1.9 ^d
Thr	4.5 ± 0.7	4.6 ± 0.4	4.5 ± 0.7	5.0	4.0	5.0	5.5 ± 0.7	5.0	4.0	5.0	4.4 ± 0.1	5.5 ± 0.7	4.9 ± 0.3	5.2 ± 0.6	4.7 ± 0.1	4.6	3.4	0.9
Val	5.0	4.9 ± 0.3	5.0	5.0	4.0	4.5 ± 0.7	5.0	5.0	4.0	5.0	4.8	5.5 ± 0.7	5.1 ± 0.5	5.1 ± 0.8	4.5 ± 0.3	3.3	3.5	1.3
Trp	2.5 ± 0.8	2.1 ± 0.4	1.9 ± 0.1	1.7 ± 0.6	2.0 ± 0.1	1.7	1.6 ± 0.1	1.6 ± 0.4	1.7 ± 0.1	2.0 ± 0.4	1.9 ± 0.4	1.7 ± 0.1	1.7	2.7 ± 0.9	1.9 ± 0.1	0.9	1.1	0.5
His	1.5 ± 0.7	1.4 ± 0.1	2.0	1.5 ± 0.7	1.0	1.0	2.0	2.0	1.0	2.0	1.3 ± 0.1	2.0	2.1 ± 0.2	1.7 ± 0.3	1.3 ± 0.2	1.7	1.9	1.6
<i>Non-essential amino acids</i>																		
Ala	3.0	3.0 ± 0.1	3.0	4.0	3.0	3.0	3.5 ± 0.7	3.0	3.0	3.5 ± 0.7	3.6 ± 0.1	3.0	3.4 ± 0.4	3.2 ± 0.2	3.5 ± 0.3	1.8		
Arg	4.5 ± 0.7	3.7	4.0	5.0	4.5 ± 0.7	4.0	3.5 ± 0.7	3.0	3.0	5.0	5.0 ± 0.3	3.5 ± 0.7	4.0 ± 0.4	4.6 ± 0.2	4.9 ± 0.8	2.6		
Asp ^e	8.5 ± 0.7	10.2 ± 1.3	9.0	10.0	11.5 ± 0.7	10.5 ± 0.7	10.0 ± 1.4	9.0	8.0 ± 2.8	11.0	10.1 ± 1.9	10.0	9.6 ± 0.1	10.2 ± 1.9	10.4 ± 0.6	5.6		
Cys-S ^f	15.5 ± 0.8	16.8 ± 1.3	14.3 ± 4.2	13.9 ± 0.2	14.1 ± 0.6	15.0 ± 1.7	13.7 ± 1.1	12.9 ± 0.4	15.0 ± 0.7	15.1 ± 0.3	14.4 ± 3.5	13.7 ± 0.9	13.3 ± 0.1	10.3 ± 1.6	17.9 ± 4.2	4.1		
Glu ^g	7.0 ± 1.4	9.8 ± 1.5	8.5 ± 0.7	9.0	9.0 ± 1.4	9.0	10.0 ± 1.4	9.0	7.5 ± 2.1	10.0	8.0 ± 1.7	11.0	10.0 ± 1.1	9.0 ± 1.2	9.2 ± 1.1	7.0		
Gly	3.0	2.8 ± 0.5	3.0	4.0	3.0	3.0	3.0	3.0	2.5 ± 0.7	4.0	3.5 ± 0.1	3.5 ± 0.7	3.8 ± 0.7	3.3 ± 0.2	3.3 ± 0.5	1.8		
Ser	4.0	4.1 ± 0.1	4.0	4.5 ± 0.7	4.5 ± 0.7	4.0	4.5 ± 0.7	4.0	3.5 ± 0.7	5.0	4.3 ± 0.4	4.5 ± 0.7	4.2 ± 0.5	4.4 ± 0.4	4.5 ± 0.3	4.3		
Tyr	2.5 ± 0.7	2.9 ± 0.8	3.0	2.0	2.5 ± 0.7	2.5 ± 0.7	2.5 ± 0.7	2.0	2.5 ± 0.7	3.5 ± 0.7	2.7 ± 0.5	3.0	2.5 ± 0.2	2.3 ± 0.2	2.4 ± 0.1	2.5		
Pro	ND ^a	ND ^a	ND ^a	ND ^a	ND ^a	ND ^a	ND ^a	ND ^a	ND ^a	ND ^a	ND ^a	ND ^a	ND ^a	ND ^a	ND ^a	3.3		

^a Not determined.

^b FAO/WHO/UNU energy and protein requirements (1985).

^c Requirements for Methionine + Cysteine.

^d Requirements for Phenylalanine + Tyrosine.

^e Aspartic acid + Asparagine.

^f Cysteine + Cystine.

^g Glutamic acid + Glutamine.

for infants, which suggested the potential of ovomucin hydrolysates as ingredients in nutritional formulations.

3.5. Peptide profiles of ovomucin hydrolysates

The peptide profiles were analyzed by reverse phase HPLC. Ovomucin hydrolysates displayed very different profiles, suggesting different cleavage sites of enzymes (Fig. 2(b)). Elution from a reverse phase column can be used as an index of the hydrophobicity; peptides prepared with pepsin-pancreatin, protease M, pronase, protease A and protease P eluted early while hydrolysates prepared by alcalase 2.4L, pepsin, protex 6L, and trypsin VI were comparatively more hydrophobic (Fig. 2(b)). Protex 26L and protease N hydrolysates were evenly distributed over the elution time. Hydrolysates prepared by pepsin, protex 26L and acid protease II showed low M_w (Fig. 2(a)) but eluted later in chromatography, suggesting high hydrophobicity, possibly related to low glycosylation.

3.6. IgE binding ability of ovomucin hydrolysates

Egg proteins are potential food allergens (Mine & Zhang, 2002). Enzymatic hydrolysis is an efficient method to reduce potential protein allergenicity (Ena, Van Beresteijn, Robben, & Schmidt, 1995), therefore the effect of hydrolysis on the allergenicity was determined. The IgE-binding abilities of ovomucin hydrolysates were determined using four sera from patients with confirmed egg allergies. All the patient sera bound the acidified egg white (positive control), and serum #14474 showed the highest binding activity (Fig. 3D). After protease digestion, the IgE binding ability of ovomucin hydrolysates were all reduced ($P < 0.05$) or even below the detection limit. Hydrolysates from flavourzyme, protex 51FP,

protex 6L, and protease A were determined to have a lower DH but retained IgE-binding activity; especially, the hydrolysate prepared by protex 6L which revealed high binding activity with both sera 17912 (Fig. 3A) and 14474 (Fig. 3D). The decreased IgE binding of ovomucin hydrolysates possibly results from the hydrolysis of epitopes by proteases (Mills, Madsen, Shewry, & Wichers, 2003). Generally, a low degree of hydrolysis corresponded to a relatively high IgE binding ability of hydrolysates.

3.7. Metabolism of ovomucin hydrolysates by bifidobacteria

Six bifidobacterial strains were fermented in media with ovomucin hydrolysate as the sole carbon source. Compared to the growth of bifidobacteria in the negative control (MRS without added sugars), only *B. infantis* displayed growth in the ovomucin medium. Among the tested ovomucin hydrolysates, and only pepsin-pancreatin hydrolysate significantly promoted the growth of *B. infantis* after 24 h of incubation and increased the production of lactate shown in Fig. 4(a and b) ($P < 0.05$).

The degradation of carbohydrates in media by bifidobacteria was also evaluated. Only galactose and mannose in pepsin-pancreatin hydrolysates were decreased by 9.5% and 14.9%,

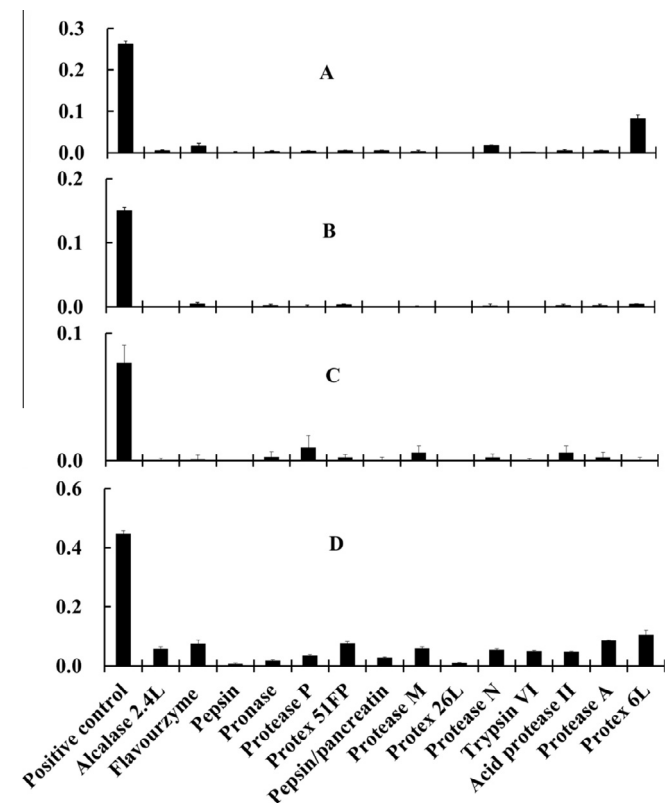


Fig. 3. IgE binding activity of ovomucin hydrolysates. The positive control was acidified egg white. The proteases in the picture represented the corresponding ovomucin hydrolysates. A: serum 17912, B: serum 12388, C: serum 14982, D: serum 14474.

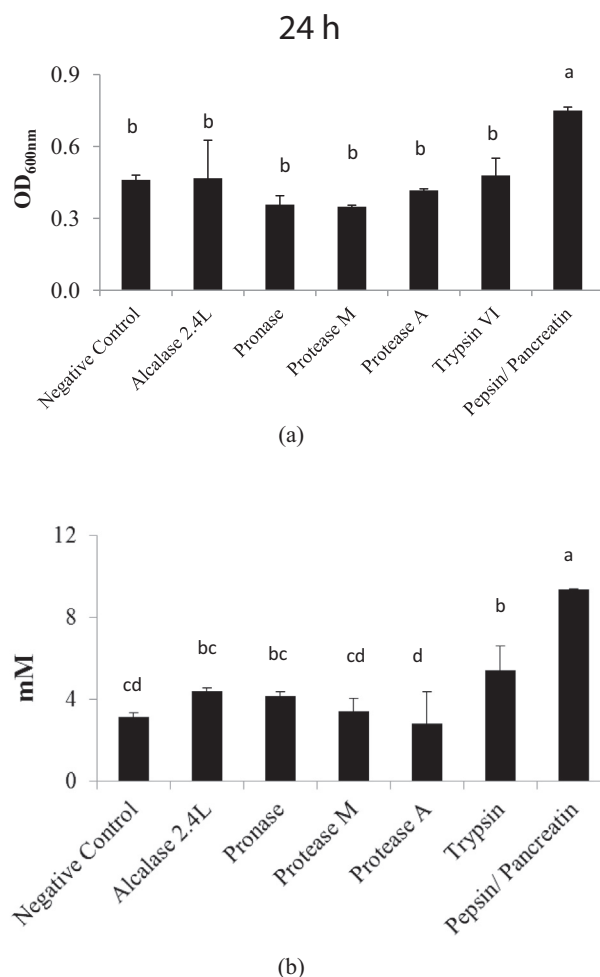


Fig. 4. (a) The OD values (600 nm) at 24 h when *B. infantis* fermented in modified MRS with ovomucin hydrolysates as sole carbohydrate source at 37 °C for 48 h under anaerobic condition (10% of CO₂, 5% of H₂, and 85% N₂). The proteases in the picture represented the corresponding ovomucin hydrolysates. The bars with different letters above were significantly different ($P < 0.05$). (b) The production of lactate (48 h minus 0 h) by the strain of *B. infantis*. The other information was same to that in Fig. 4(a).

respectively, after fermentation by *B. infantis*. Glycopeptides present in the ovomucin hydrolysate were thus only partially used as a carbon source by *B. infantis*. Firstly, ovomucin is rich in sialic acid, which occupies the terminal position of the glycans within ovomucin, and could resist the proteolytic effect and inhibit the action of some endoglycosidases (Schauer, 2004). Among the six tested strains, only *B. infantis* was reported to have gene clusters encoding sialidases to release the sialic acid (LoCascio et al., 2007), and secrete fucosidase, β -galactosidase and β -N-acetylhexosaminidase as well (Kitaoka, 2012). Some species of *Bifidobacterium* have been demonstrated to metabolize human milk oligosaccharides (HMO) as the sole carbon source, such as *B. bifidum* and *B. infantis* (Asakuma et al., 2011), and *B. bifidum* was clarified to degrade HMO by α -fucosidases (Ashida et al., 2009). The structures of ovomucin glycans differ substantially from HMO; in particular, ovomucin contains no fucose. Enzymes produced by bifidobacteria for degradation of HMO (Sela & Mills, 2010) may thus not hydrolyze glycans from ovomucin. It is likely, however, that ovomucin is fermented in the large intestine by other members of the intestinal microbiota (Klaassens et al., 2009; Sela & Mills, 2010).

4. Conclusions

Ovomucin extract was hydrolyzed by 14 proteases within 4 h, in comparison to 24 h as reported in literature. Yields and DHs ranged from 42.6% (flavourzyme) to 97.4% (protease N), and 2.4% (flavourzyme) to 46.3% (pronase), respectively. These hydrolysates showed a wide range of M_w distribution while only two of the hydrolysates prepared by pronase and protex 26L showed M_w less than 40 kDa. The content of sialic acid in these hydrolysates ranged from 0.1% (protex 26L) to 3.7% (pronase). IgE binding of ovomucin hydrolysates was significantly reduced ($P < 0.05$) in comparison to ovomucin extracts. Ovomucin hydrolysates did not generally support growth of *Bifidobacterium* spp. *in vitro*, but may be degraded within the infant gastrointestinal tract by potential protocoooperation among different bifidobacterial strains or other members of the gut microbiota and form short chain fatty acid.

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