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Characterization of novel galactosylated chitin-oligosaccharides and chitosan-oligosaccharides

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ABSTRACT

This study determined whether chitin-oligosaccharides and chitosan-oligosaccharides (COS) are suitable acceptor carbohydrates for the LacLM-type β-galactosidase of *Lactobacillus plantarum*. Enzymatic reactions were carried out with lactose as galactosyl-donor and chitinbiose (N,N′-diacetyl chitobiose), chitintriose (N,N′N″-triacetyl chitotriose) or a highly deacetylated COS preparation with a degree of polymerization ranging from 2 to 4 as galactosyl acceptors. Transgalactosylated oligosaccharides were identified by liquid chromatography-mass spectrometry (LC-MS/MS). LC-MS/MS analysis demonstrated formation of mono- and digalactosylated chitinbiose, mono-galactosylated chitintriose, and mono-, di-, and trigalactosylated COS. β-(1→4)-Linkages were formed during the galactosylation of chitinbiose and chitintriose. In conclusion, the conversion of lactose with β-Gal from *Lb. plantarum* and chitin-oligosaccharides or COS as glycosyl acceptors allows the production of novel oligosaccharides.

1. Introduction

The valorization of lactose in whey remains a challenge for the dairy industry (Gänzle, Haase, & Jelen, 2008). The conversion of lactose to oligosaccharides is one of the promising avenues to convert lactose to food ingredients with desirable technological attributes or health benefits (Díez-Municio, Herrero, Olano, & Moreno, 2014; Gänzle, 2012). Desirable attributes of lactose-derived oligosaccharides include sweet taste, low caloric value, prebiotic properties, and the inhibition of pathogen adhesion (Díez-Municio et al., 2014; Gänzle, 2012). Several of these properties, particularly the inhibition of pathogen adhesion, are highly dependent on the structure and composition of oligosaccharide preparations (Gänzle, 2012). Moreover, the commercial application of novel oligosaccharides necessitates efficient methods for production (Díez-Municio et al., 2014).

The commercial production of galacto-oligosaccharides (GOS) predominantly relies on β-galactosidases from fungi and *Bacillus circulans*, but enzymes from lactobacilli were suggested as a suitable alternative for production of GOS (Black et al., 2012; Nguyen et al., 2012). Lactobacilli have a safe history of food use (Hammes & Hertel., 2009); moreover, GOS produced by the heterodimeric LacLM-type β-galactosidases (β-Gal) from lactobacilli differ in their composition from commercial GOS (Black et al., 2012; Nguyen et al., 2012; Urrutia et al., 2013). β-Galactosidases from lactobacilli were particularly employed for the production of novel hetero-oligosaccharides (Gänzle, 2012). LacLM-type β-Gal from *Lactobacillus plantarum* formed β-(1→4)- and β-(1→6)- linked oligosaccharides when N-acetylglucosamine (GlcNAc) is added as galactosyl-acceptor (Black et al., 2012). GlcNAc is the constituent monosaccharide of chitin and transgalactosylation of GlcNAc or GlcNAc-oligosaccharides may increase the range of products produced from lactose and incorporate properties of chitin-oligosaccharides that are outlined below.

Chitin is a linear polymer of β-(1→4)-linked GlcNAc. In industrial processes, it is extracted from shellfish waste (Mathur & Narang, 1990). Commercial applications of chitin are limited by its poor solubility (Mathur & Narang, 1990); however, chitosan, the deacetylated form of chitin, is more soluble than chitin (Qin et al., 2006) and can be enzymatically or chemically depolymerized to form chitosan-oligosaccharides (COS) with a degree of polymerization of 2–10 (Jeon & Kim, 2000; Kaur & Dhillon, 2013). Chitosan and COS are inexpensive to produce and possess multiple biological activities. The antibacterial and antifungal activity of chitosan are relevant for food applications (Helander, Nurmiaho-Lassila,

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Ahvenainen, Rhoades, & Roller, 2001; Kong, Chen, Xing, & Park, 2010; Liu, Du, Wang, & Sun, 2004; Mellegård, Strand, Christensen, Granum, & Hardy, 2011). Chitosan preparations with a molecular mass ranging from 10 to 200 kDa exhibit antibacterial activity (Cai et al., 2010; Kong et al., 2010; Mellegård et al., 2011).

COS also promote growth of some beneficial gastrointestinal microbiota in vitro (Lee, Park, Jung, & Shin, 2002). COS also inhibit the adhesion of enteropathogenic *Escherichia coli* (EPEC) by interfering with bacterial-host cell interactions as receptor analogues (Quintero-Villegas et al., 2013). Such receptor analogues prevent viruses, pathogenic bacteria and bacterial toxins from interacting with the surface glycans of eukaryotic cells, by acting as molecular decoys for adhesion, and thereby prevent infection. Because β-glycosylated GlcNAc moieties often occur at the reducing end of mammalian cell surface glycans, similarly structured β-glycosylated-GlcNAc oligosaccharides act as receptor analogues. For example, N-acetyllactosamine, Galβ-(1→4)-GlcNAc, the core structure of human milk oligosaccharides, was identified as a competitive inhibitor to EPEC (Hyland et al., 2008). Galβ-(1→4)-GlcNAc also acts as a receptor for *Pseudomonas aeruginosa*, *Salmonella typhimurium* and *Neisseria gonorrhoea* (Ramphal et al., 1991; Shoaf-Sweeney & Hutkins, 2008).

It follows, therefore, that galactosylation of GlcNAc or chitin-oligosaccharides may increase their activity as receptor analogues to prevent adhesion of a wide range of bacterial pathogens. However, it remains undetermined as to whether the degree of polymerization of the acceptor carbohydrates influences transgalactosylation by galactosidases. Moreover, current processes for the extraction and conversion of chitin produce glucosamine or chitosan and COS with a high degree of deacetylation (Kaur & Dhillon, 2013; Vaaje-Kolstad, Horn, Sørlie, & Eijsink, 2013) but glucosamine or corresponding oligosaccharides have not been employed as acceptor carbohydrates for enzymatic transgalactosylation. Therefore, it was the aim of this study to determine if chitin-oligosaccharides and chitosan-oligosaccharides are galactosylated by the transglycosylation reaction of β-galactosidases of lactobacilli. The chitin-oligosaccharides chitinbiose (N,N′-diacetyl chitobiose), chitintriose (N,N′N″-triacetyl chitotriose), chitinpentaose, and a highly deacetylated COS preparation with a DP ranging from 2 to 4 were employed as galactosyl acceptors. Transgalactosylated oligosaccharides were identified by liquid chromatography-mass spectrometry (LC/MS) as described (Black et al., 2012; Wang, Black, Curtis, & Gänzle, 2014).

2. Materials and methods

2.1. Chemicals and standards

The oligosaccharide standards β-(1→3) galactosyl-N-acetyl glucosamine [lacto-N-biose, Galβ-(1→3)-GlcNAc], β-(1→4) galacto syl-N-acetyl glucosamine [N-acetyl-D-lactosamine, Galβ-(1→4) GlcNAc], β-(1→6) galactosyl-N-acetyl glucosamine [Galβ-(1→6)-GlcNAc], chitinbiose, chitintriose, and chitinpentaose were purchased from Dextra Laboratories (Reading, UK). GlcNAc, *o*-nitrophenyl-β-galactoside, DNase I from bovine pancreas and protease inhibitor cocktail were purchased from Sigma Aldrich (Oakville, Canada). COS (90% deacetylated) were enzymatically prepared from shrimp shells and were provided by Yumin Du, Department of Environmental Science at the University of Wuhan (Wuhan, China). Fisher Scientific (Ottawa, Canada) supplied HPLC grade acetonitrile, methanol, and ammonium acetate. Other solvents were of analytical grade.

2.2. Bacterial strains and preparation of crude cell extract

Lb. plantarum FUA3112 was grown under microaerophilic conditions (1% O_2, balance N_2) at 30 °C in modified DeMan-Rogosa-Sharpe (mMRS) broth containing 2% (w/v) lactose with a pH of 6.2. *Lactococcus lactis* MG1363, a β-Gal negative strain, was grown in a modified M17 (mM17) broth with the addition of 0.5% (w/v) glucose at 30 °C. *Lc. lactis* MG1363 harboring pAMJ586 with LacLM from *Lb. plantarum*, as the sole source of β-Gal activity (Schwab, Sørensen, & Gänzle, 2010) was grown in modified M17 with addition of 5 mg L^{-1} erythromycin. Overnight cultures from single colonies were used to inoculate 500 mL mMRS or mM17 broth at a 1% (v/v). Cultures were incubated until the medium was acidified to pH 5.0 to 5.2. Cultured cells were harvested and washed twice before suspension in 1 mL of 50 mM sodium phosphate buffer (pH 6.5) with 10% glycerol and 1 mM magnesium chloride. The cell suspension was transferred to screw-cap tubes with 0.5 g of Zirconia/Silica beads (0.1 mm), disrupted in a Mini Beadbeater-8 (model 693, BioSpec, Bartlesville, OK, USA) for 2 min, and chilled in ice for a minimum of 5 min. The supernatant was collected by centrifugation of disrupted cells (15,300 × *g* for 10 min at 4 °C) and designated crude cell extract (CCE). The CCEs of *Lb. plantarum*, *Lc. lactis* and *Lc. lactis* expressing LacLM from *Lb. plantarum*, were collected from three independent culture fermentations and used for oligosaccharide synthesis. The protein content of CCEs was determined with the Bio-Rad protein assay reagent (Bio-Rad, Mississauga, Canada). *o*-Nitrophenyl-β-galactoside was used to determine specific β-galactosidase activity of CCEs as described (Schwab et al., 2010). Similar to previous studies synthesizing oligosaccharides using β-Gal, activities of CCE ranged from 25 to 30 mmol min^{-1} mg $protein^{-1}$ (Black et al., 2012; Schwab, Lee, Sørensen, & Gänzle, 2011; Schwab et al., 2010). CCE of *Lc. lactis* MG1363 displayed no β-Gal activity and was used as a negative control.

2.3. Synthesis of galactosylated chitin-oligosaccharides and chitosan-oligosaccharides

To prepare saccharide solutions for reactions lactose, GlcNAc, COS, chitinbiose, and chitintriose and chitinpentaose were dissolved into 50 mM potassium phosphate buffer (pH 6.8) with 100 mM potassium chloride and 2 mM magnesium chloride at 90 °C. COS were dissolved at 50 °C. The addition of 10% ethanol was used to improve the solubility of chitinbiose and chitintriose without impacting enzyme activity (data not shown). Lactose was used at a concentration of 1 M to produce galacto-oligosaccharides (GOS) with 20% CCE of *Lb. plantarum*, *Lc. lactis* and *Lc. lactis* expressing LacLM from *Lb. plantarum* in three separate reactions. Similarly, acceptor reactions were performed at a 1 M total saccharide concentration of 1:1 (w/w) lactose-acceptor saccharide with 20% CCE of *Lb. plantarum*, *Lc. lactis* and *Lc. lactis* expressing LacLM from *Lb. plantarum*. All reactions were conducted at 45 °C for 16 h and terminated by heating to 95 °C for 10 min. Experiments were conducted in triplicate.

2.4. High performance anion exchange chromatography with pulsed amperometric detection

Oligosaccharide analyses were performed using a HPAEC ICS-3000 system (Dionex, Oakville, Canada). Sample oligosaccharide solutions at 10 g L^{-1} were injected in 10 μL aliquots on to a CarboPac PA-20 Dionex carbohydrates column (3 × 150 mm). The eluents used were water (A), 0.2 M sodium hydroxide (B), and 1 M sodium acetate (C) and were used at flow rate of 0.25 mL min^{-1}. Oligosaccharides were analyzed using gradient starting at 30.4% B, 1.3% C and gradually increasing to 30.4% B, 11.34% C at 22 min.

2.5. Combined liquid chromatography/electrospray ionization tandem mass spectrometry

Oligosaccharides were analyzed by liquid chromatography/electrospray ionization tandem mass spectrometry (LC/ESI-MS/MS)