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 – 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 *L. plantarum* and chitin-oligosaccharides or COS as glycosyl acceptors allows the production of novel oligosaccharides.

1. Introduction

The valorisation 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 (Gänzle, 2012; Díez-Municio, Herrero, Olano, & Moreno, 2014). Desirable attributes of lactose-derived oligosaccharides include sweet taste, low caloric value, prebiotic properties, and the inhibition of pathogen adhesion (Gänzle, 2012; Díez-Municio et al., 2014). 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 (Nguyen et al., 2012, Black et al., 2012). Lactobacilli have a safe history of food use (Hammes & Hertel., 2009); moreover, GOS produced by the heterodimeric LacLM-type β-galactosidases 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 *L. 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 (Liu, Du, Wang, & Sun, 2004; Kong, Chen, Xing, & Park, 2010; Mellegård, Strand, Christensen, Granum, & Hardy, 2011; Helander, Nurmiaho-Lassila, Ahvenainen, Rhoades, & Roller, 2001). Chitosan preparations with a molecular weight ranging from 10 to 200 kDa exhibit antibacterial activity (Kong et al., 2010; Cai 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 *E. 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). Gal*β*-(1→4)-GlcNAc also acts as a receptor for *Pseudomonas aeruginosa*, *Salmonella typhimurium* and *Neisseria gonorrhoea* (Ramphal et al., 1991; Shoaf-Sweeney, & Hutkins, 2008). Hence, 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 – 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, Gal*β*-(1→3)-GlcNAc), *β*-(1→4) galactosyl-N-acetyl glucosamine (N-acetyl-D-lactosamine, Gal*β*-(1→4)GlcNAc), β-(1→6) galactosyl-N-acetyl glucosamine (Gal*β*-(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

Lactobacillus 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 *L. 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, U.S.A.) 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 *L. plantarum*, *Lc. lactis* and *Lc. lactis* expressing LacLM from *L. 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 – 30 mmol (min × mg protein)$^{-1}$ (Schwab et al., 2010; Black et al., 2012; Schwab, Sørensen, & Gänzle, 2011). 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 *L. plantarum*, *Lc. lactis* and *Lc. lactis* expressing LacLM from *L. 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 *L. plantarum*, *Lc. lactis* and *Lc. lactis* expressing LacLM from *L. 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) were used at flow rate of 0.25 mL min^{-}

[1]. Oligosaccharides were analysed 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 analysed by liquid chromatography / electrospray ionization tandem mass spectrometry (LC/ESI-MS/MS) for structural characterization. Separations were conducted on an Agilent 1200 series LC system (Agilent Technologies, Palo Alto, CA, U.S.A.) at 60 °C using a Supelcosil LC-NH_2 column (250 mm x 4.6 mm i.d., 5 μm; Sigma Aldrich, Oakville, Canada). Analytes were adjusted to a final concentration of approximately 0.1 g L^{-1} carbohydrates in water, with the exception of quasi-MS^3 experiments (see below); in these cases the concentration was increased to 1 g L^{-1} and analysed with an injection volume of 25 μL. Samples were eluted with acetonitrile (solvent A) and water (solvent B) with at a flow rate of 1.5 mL min^{-1} and the following gradient: 0 min, 20%B; 20 min, 25% B, 35 min, 40%B, followed by re-equilibration of the column. The effluent from the column was split to obtain a flow rate to the mass spectrometer of 0.4 mL min^{-1}. A 10% (v/v) post-column addition of 40 mM ammonium acetate in methanol was delivered by an Agilent 1200 series isocratic pump to the flow entering the ESI source.

Mass spectrometry was performed on a QStar® Elite hybrid orthogonal Q-TOF mass spectrometer coupled to a TurboIon Spray® source with Analyst® QS 2.0 software (Applied Biosystems/MDS Sciex, Concord, Canada). The source conditions for negative ion MS were as follows: nebulizer gas, 50 (arbitrary units); auxillary gas, 60 (arbitrary units); ionspray voltage, -4500 V; curtain gas, 25 (arbitrary units); source temperature, 400 °C; declustering potential, -50 V; focusing potential, -190 V; declustering potential-2, -10 V and a mass range of *m/z* 100-900. Fragmentation was achieved using nitrogen as a collision gas at a collision energy that varied

between -10 to -25 eV, decreasing in energy as the degree of polymerization increased. Quasi MS^3 spectra were obtained from the MS/MS spectra of in-source fragment ions formed by increasing the declustering potential by an additional 30 V.

3. Results

3.1. Identification of galactosylated chitin-oligosaccharides

The formation of higher oligosaccharides via enzymatic galactosylation reactions was initially verified by HPAEC-PAD (data not shown). This included lactose and GlcNAc, which were previously shown to act as galactosyl-acceptors for the LacLM-type *β*-galactosidase of *L. plantarum* (Black et al., 2012), as positive controls. Reactions with CCE of *Lc. lactis* MG1363 without *β*-Gal activity served as negative control to consider only peaks resulting from the highly specific β-galactosyl transfer of β-Gal (Juers, Matthews, & Huber, 2012). Then, LC/ESI-MS experiments were performed to measure the elution times and exact masses of the galactosylated products formed from *β*-transgalactosylation of lactose, GlcNAc, chitinbiose, chitintriose and COS (Figure 1 and Table 1). The *β*-Gal reactions with lactose, and the *β*-Gal reactions with GlcNAc plus lactose, formed GOS and galactosylated-GlcNAc oligosaccharides, respectively, as previously described (Black et al., 2012). The analysis of all of the products by LC/ESI-MS also demonstrated the presence of galactosylated chitinbiose, chitintriose (Figure 1A and 1B, respectively), and COS. The initial characterization of products by chromatographic separation and by the accurate determination of the *m/z* for $[M-H]^-$ ions demonstrated that two products were formed with chitinbiose as a galactosyl-acceptor and one product with chitintriose as a galactosyl-acceptor (Table 1). Chitin-oligosaccharides and COS thus act as acceptors for LacLM–type *β*-Gal from *L. plantarum*. Reactions with crude cellular extract from *Lc. lactis* MG1363 formed no identifiable hydrolyzed- or galactosylated-products, which confirms that

transgalactosylation is attributable to β-galactosidase activity. Chitinpentaose remained largely insoluble and hence did not yield detectable quantities of galactosylated chitinpentaose.

3.2. Structure elucidation of galactosylated chitin-oligosaccharides

Tandem MS (MS/MS) and quasi-MS^3 experiments were performed on galactosylated-chitinbiose and galactosylated-chitintriose in order to determine their monosaccharide sequences and the position of their glycosidic bonds. It was found that the trisaccharide eluting at 10.4 min, formed from chitinbiose as a galactosyl-acceptor, presented *m/z* 179 and 382 C-type glycosidic cleavages in the MS/MS spectra, confirming the monosaccharide sequence Gal-GlcNAc-GlcNAc (Figure 2A). Additionally, $^{0,2}A$ and $^{0,2}A(-H_2O)$ fragments, along with the absence of a $^{0,3}A$ cross-ring fragment, indicate the presence of 1→4 linkages (Black et al., 2012). To further confirm the linkage formed by *β*-Gal in the transfer of galactose, the ion-source induced C_2 fragment ion at *m/z* 382 was further fragmented in a quasi-MS^3 experiment (Figure 2B). Similar to the MS/MS spectrum of the trisaccharide molecular ion (Figure 2A), $^{0,2}A$ and $^{0,2}A(-H_2O)$ fragment ions were formed with an absence of an $^{0,3}A$ fragment ion. Furthermore, the *m/z* 382 fragmentation from the quasi MS^3 experiment matched the fragmentation of an authentic Gal*β*-(1→4)-GlcNAc standard (Black et al., 2012). In summary, the compound that eluted at 10.4 min with a *m/z* 585.2149 was identified as the product of *β*-transgalactosylation of chitinbiose with the structure Gal*β*-(1→4)-GlcNAc*β*-(1→4)-GlcNAc. Products eluting at 16.1 and 17.1 min were identified as tetrasaccharides with the sequence Gal-Gal-GlcNAc-GlcNAc on the basis of the *m/z* of 747.2692. However, the abundance of these *m/z* ions was too low to obtain reliable MS^2 spectra for linkage determination (Figure 1A). Equivalent results were obtained with *L. plantarum* and *L. lactis* expressing LacLM of *L. plantarum* (data not shown).

Compounds formed with chitintriose as a galactosyl-acceptor in *β*-Gal reactions were also analyzed via MS/MS and quasi-MS^3 experiments. The C-type fragments arising from the tandem mass spectra of Gal–$(GlcNAc)_3$ confirm part of the monosaccharide sequence (Figure 3A). Moreover, the accompanying A-type cross-ring fragments of $^{0,2}A$ and $^{0,2}A(-H_2O)$, along with the absence of an $^{0,3}A$ fragment, confirmed all of the linkages with exception of the Gal-GlcNAc linkage. In order to determine the remaining structural information, a quasi MS^3 experiment was completed, again by selecting the C_2 fragment ion at *m/z* 382 for further MS/MS analysis (Figure 3B). The sequence order for this disaccharide fragment could then be deduced from the resulting C_1 fragment ion at *m/z* 179, confirming the galactose moiety at the non-reducing end. The MS/MS fragmentation of the ion-source derived C_2 fragment ion at *m/z* 382 yielded the A-type cross-ring fragments $^{0,2}A$ and $^{2,4}A$ (Figure 3B), which are indicative of a 1→4 linkage. Hence, the compound produced by *β*-transgalactosylation of chitintriose, which elutes at 15.7 min and has an $[M-H]^-$ ion at *m/z* 788.2942, was identified as Gal*β*-(1→4)-GlcNAc*β*-(1→4)-GlcNAc*β*-(1→4)-GlcNAc (Figure 3B). This trisaccharide was obtained in reactions containing crude cellular extracts from *L. plantarum* as well as crude cellular extracts of *L. lactis* expressing LacLM from *L. plantarum* (data not shown).

3.3. Characterization of galactosylated chitosan-oligosaccharides

HPAEC-PAD and LC/MS analyses indicated that the COS preparation employed as galactosyl-acceptor contained oligosaccharides composed of glucosamine and N-acetylglucosamine with a DP of 2, 3 and 4; glucosamine as additionally detected (Table 1 and data not shown). These analyses also confirmed a high degree of deacetylation (Table 1). The LC/MS exact mass determination for the oligosaccharide $[M-H]^-$ ions demonstrated that COS were galactosylated irrespective of them being acetylated, demonstrating that glucosamine and COS act as

galactosyl-acceptors for β-Gal (Table 1). Pentasaccharides in the products included Gal_3-$GlcN_2$ and mono-galactosylated COS. In contrast to the galactosylated GlcNAc (Black et al., 2012) and chitinbiose (Table 1), only one peak with the same *m/z* was detected. As a consequence of the diversity of acceptor carbohydrates, the abundances of molecular ions for individual galactosylated GOS observed in LC/MS experiments were inadequate for further MS/MS experiments.

4. Discussion

The production of functional oligosaccharides has substantial economic potential for valorization of lactose from whey and other lactose-enriched by-products of milk fractionation (Gänzle et al., 2008). The production of functional glycoconjugates via Maillard-chemistry was proposed (Seo, Karboune, & Archelas, 2014) but the enzymatic production of oligosaccharides remains the main route of oligosaccharide production from lactose (Urrutia et al., 2013; Díez-Municio et al., 2014). Health benefits of GOS are based on prebiotic properties and colonic fermentation (Venema, 2012); additional health benefits are based on oligosaccharide-mediated modulation of the immune response (Meijerink et al., 2012; Li et al., 2014) and the prevention of pathogen adhesion (Shoaf-Sweeney & Hutkins, 2008). The prevention of pathogen adhesion by oligosaccharides adhesion is highly specific for the oligosaccharide structure (Shoaf-Sweeney & Hutkins, 2008); synthesis of oligosaccharide arrays to target a broad range of pathogens thus necessitates novel tools for enzymatic oligosaccharide synthesis (Díez-Municio et al., 2014).

Galactosylated- GlcNAc oligosaccharides are often targets for different biological functions, including recognition by pathogenic toxins, microorganisms and antibodies (Varki, 1993; Kunz, Rudloff, Baier, Klein, & Strobel, 2000; Ofek, Bayer, & Abraham, 2013). Chemically galactosylated chitosan acted as strong immunological stimulant without toxicity to

rats (Song et al., 2009). Galactosylated GlcNAc can be produced with α-Gal or β-Gal from lactobacilli and GlcNAc as acceptor (Black et al., 2012; Wang et al., 2014). However, GlcNAc is relatively expensive when compared to chitosan or COS produced from by-products of shellfish processing (Jeon & Kim, 2000). This study employed β-Gal of *L. plantarum* to galactosylate chitin-oligosaccharides and COS. LC/MS was used to confirm that chitinbiose, chitintriose and COS were suitable acceptors in *β*-Gal transgalactosylation reactions. Galactosylated chitinbiose and -chitintriose were identified indicating that oligosaccharides as well as GlcNAc monomers act as galactosyl-acceptors in *β*-Gal reactions.

From LC/MS/MS analyses, LacLM-type *β*-Gal from *L. plantarum* formed specifically *β*-(1→4)-linkages during the galactosylation of chitinbiose and chitintriose. The *β*-(1→4)-linkage of Gal*β*-chitinbiose and -chitintriose contrasts the predominant *β*-(1→6)-linkage formed with the monomers GlcNAc or galactose as a galactosyl-acceptor of *β*-Gal (Black et al., 2012). The linkage type of GOS and hetero-oligosaccharides is known to be influenced by properties of the β-galactosidase employed in transglycosylation (Gänzle, 2012). This study confirms and extends a prior report indicating that properties of the acceptor carbohydrate may influence the linkage type formed in transglycosylation by β-galactosidases (Zheng, Yoshino, Murata, Ajisaka, Usui, 2000). Anti-adhesion activity has particularly been demonstrated for Gal-*β*-(1→4)-linked GlcNAc (Ramphal et al., 1991; Hyland et al., 2008; Shoaf-Sweeney, & Hutkins, 2008). Galactosylation of chitinbiose rather than GlcNAc may thus facilitate the formation of *β*-(1→4)-linked oligosaccharides.

The yield of transgalactosidation by β-Gal is dependent on properties of the specific acceptor carbohydrate (Gänzle, 2012). We detected galactosylated COS ranging from DP1 to DP5 with various degrees of acetylation, demonstrating that the deacetylation of COS did not

prevent galactosylation. During β-Gal reactions, the amino groups of COS were predominantly uncharged because the pH of the buffer for enzymatic reactions, 6.8, was well above the pKa of COS, 5.5. The linkage type of galactosylated COS could not be determined; however, it was ascertained that only one linkage type was formed. Because the N-acetylation had no detectable influence on the galactosylation of COS or chitin-oligosaccharides, the COS were presumably β-(1→4)-linked.

GOS and COS are known to prevent adhesion of enteropathogenic *E. coli* (Shoaf-Sweeney & Hutkins, 2008; Quintero-Villegas et al., 2013); however, EPEC and ETEC employ different mechanisms of adhesion and different glycan-binding proteins (Hyland et al., 2008; Kaper, Nataro, & Harry, 2004). In keeping with a specific and high activity of COS against ETEC K88, COS at a dose of 0.16 g kg feed^{1} reduced the duration and severity of diarrhea in ETEC K88 challenged piglets (Liu et al., 2010). However, the determination of the specific activity of galactosylated COS remains subject to further work with purified fractions or compounds.

In conclusion, the conversion of lactose with LacLM-type β-Gal from *L. plantarum* and chitin-oligosaccharides or COS as glycosyl acceptors allows the production of novel oligosaccharides. This is the first report on the formation of galactosylated-chito-oligosaccharides. Galactosylation of COS increases the structure variation for functional components that can be used for novel application. Complementation of transgalactosylation with β-Gal with the transglycosylation by chitinases (Zakariassen, Hansen, Jøranli, Eijsink, & Sørlie, 2011) may further extend the range of specific glycans that can be produced by (chemo)-enzymatic synthesis from inexpensive and abundant substrates.

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FIGURE LEGENDS

Figure 1. Liquid chromatography/mass spectrometry extracted ion chromatograms of galactosylated chitin-oligosaccharide $[M-H]^-$ ions. (A) chitinbiose as galactosyl-acceptor *m/z* 585 Gal-GlcNAc-GlcNAc (grey) and *m/z* 747 Gal-Gal-GlcNAc-GlcNAc (black); and (B) chitintriose as galactosyl-acceptor *m/z* 788 Gal-GlcNAc-$(GlcNAc)_2$

Figure 2. Mass spectra of sample compound Gal*β*-(1→4)-GlcNAc*β*-(1→4)-GlcNAc with elution time of 10.4 min as obtained by electron spray ionization-tandem mass spectrometry. (A) $[M-H]^-$ ion at *m/z* 585.2149 ($C_{22}H_{37}O_{16}N_2$); (B) In-source fragment ion C_2 at *m/z* 382.1355 ($C_{14}H_{24}O_{11}N$). Equivalent results were obtained with *L. plantarum* and *L. lactis* expressing LacLM of *L. plantarum*.

Figure 3. Mass spectra of sample compound Gal*β*-(1→4)-GlcNAc*β*-(1→4)-GlcNAc*β*-(1→4)-GlcNAc with elution time of 15.7 min as obtained by electron spray ionization-tandem mass spectrometry. (A) $[M-H]^-$ ion at *m/z* 788.2942 ($C_{30}H_{50}O_{21}N_3$); (B) In-source fragment ion C_2 at *m/z* 382.1355 ($C_{14}H_{24}O_{11}N$).). Equivalent results were obtained with *L. plantarum* and *L. lactis* expressing LacLM of *L. plantarum*.

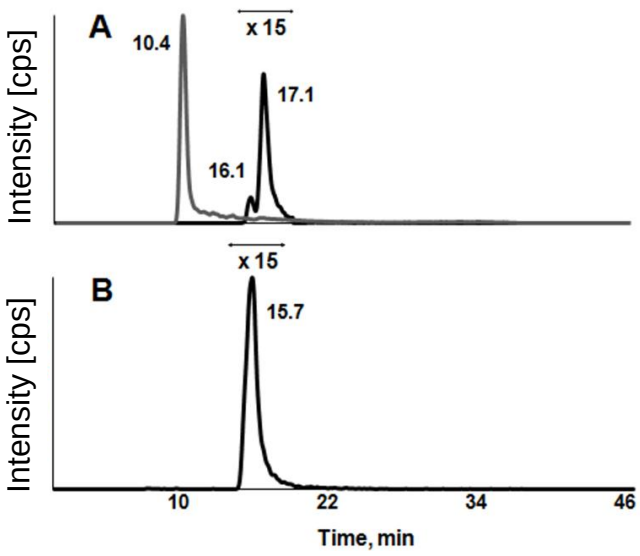
Table 1. Deprotonated molecular ions and retention times of galactosylated oligosaccharides and their mass accuracy. Galactosylated oligosaccharides were produced with *L. lactis* expressing LacLM of *L. plantarum*.

Acceptor	Oligosaccharide[a]	Retention Time (min)	Measured Mass (Da)	Error (mDa)
Chitinbiose	Gal – GlcNAc – GlcNAc	10.4	585.2148	-0.06
	Gal – Gal – GlcNAc – GlcNAc	16.1; 17.1	747.2692	1.51
Chitintriose	Gal – GlcNAc – GlcNAc– GlcNAc	15.7	788.2942	-0.03
COS[b]	Gal – GlcN	8.0	340.1235	-1.41
	Gal – Gal – Gal – GlcN	25.1	664.2301	-0.47
	Gal – GlcN – GlcN	14.8	501.1931	-0.63
	Gal – Gal – GlcN – GlcN	26.2	663.2466	0.05
	Gal – Gal– Gal – GlcN – GlcN	27.6	825.2933	-2.05
	Gal – GlcN – GlcNAc	11.0	543.2030	-1.29
	Gal – GlcN – GlcN – GlcN	29.3	662.2623	-0.24
	Gal – GlcN – GlcN – GlcNAc	20.4	704.2745	1.40
	Gal – Gal – GlcN – GlcN – GlcNAc	26.2	866.3223	-3.62
	Gal – GlcN – GlcN – GlcN – GlcN	31.6	823.3326	1.26
	Gal – Gal – GlcN – GlcN – GlcN – GlcN	30.8	824.3154	0.04

[a]Nomenclature: Galactose, Gal; N-acetylglucosamine, GlcNAc; N-glucosamine, GlcN; Chito-oligosaccharides, COS
[b]compounds are arranged by increasing degree of galactosylation of the same acceptor carbohydrate.

460

461 **Figure 1.**



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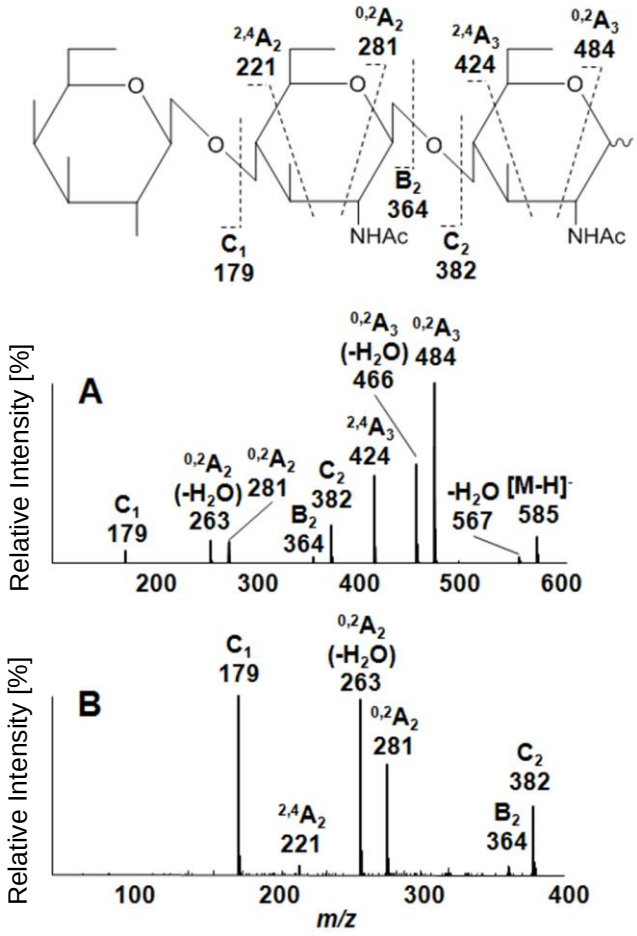
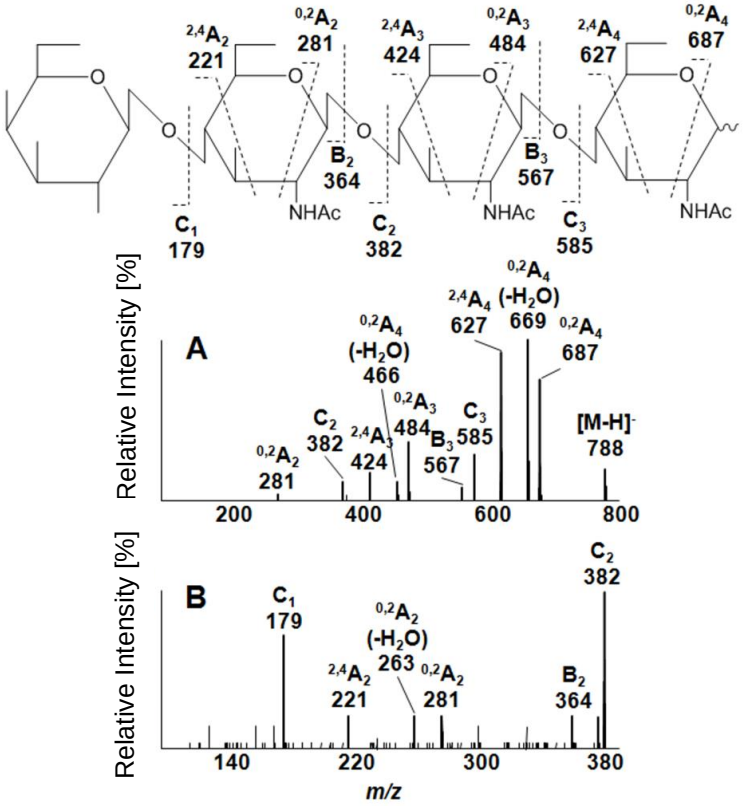


464

465 **Figure 2.**

466



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469 **Figure 3.**