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A Diverse Range of Bacterial and Eukaryotic Chitinases Hydrolyzes the LacNAC (Galβ1–4GlcNAC) and LacdiNAC (GalNAcβ1–4GlcNAC) Motifs Found on Vertebrate and Insect Cells

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Background: The biological role(s) of chitinases other than hydrolysis/metabolism of chitin is currently not well understood.

Results: A Salmonella chitinase binds N-acetylactosamine (LacNAC), a common component of mammalian glycoconjugates. Furthermore, five bacterial and eukaryotic chitinases hydrolyze terminal LacNAC and LacdiNAC from model substrates.

Conclusion: LacdiNAC and LacNAC- glycans are substrates for chitinases.

Significance: Vertebrate and invertebrate molecules carrying LacNAC and LacdiNAC motifs are potential chitinase targets.

There is emerging evidence that chitinases have additional functions beyond degrading environmental chitin, such as involvement in innate and acquired immune responses, tissue remodeling, fibrosis, and serving as virulence factors of bacterial pathogens. We have recently shown that both the human chitotriosidase and a chitinase from Salmonella enterica serovar Typhimurium hydrolyze LacNAC from Galβ1–4GlcNACβ-tetramethylrhodamine (LacNAC-TMR (Galβ1–4GlcNACβ(CH2)nCONH(CH2)2NHCOTMR)), a fluorescently labeled model substrate for glycans found in mammals. In this study we have examined the binding affinities of the Salmonella chitinase by carbohydrate microarray screening and found that it binds to a range of compounds, including five that contain LacNAC structures. We have further examined the hydrolytic specificity of this enzyme and chitinases from Sodalis glossinidius and Polysphondylium pallidum, which are phylogenetically related to the Salmonella chitinase, as well as unrelated chitinases from Listeria monocytogenes using the fluorescently labeled substrate analogs LacdiNAC-TMR (GalNAcβ1–4GlcNACβ-TMR), LacNAC-

TMR, and LacNACβ1–6LacNAcβ-TMR. We found that all chitinases examined hydrolyzed LacdiNAC from the TMR aglycone to various degrees, whereas they were less active toward LacNAC-TMR conjugates. LacdiNAC is found in the mammalian glycome and is a common motif in invertebrate glycans. This substrate specificity was evident for chitinases of different phylogenetic origins. Three of the chitinases also hydrolyzed the β1–6 bond in LacNACβ1–6LacNAcβ-TMR, an activity that is of potential importance in relation to mammalian glycans. The enzymatic affinities for these mammalian-like structures suggest additional functional roles of chitinases beyond chitin hydrolysis.

Chitinases are ubiquitous enzymes found in archaea, bacteria, fungi, plants, and animals. They hydrolyze chitin, the second most abundant polysaccharide in nature. Chitin is a linear polymer comprised of β1–4-linked N-acetyl-

N-glucosamine (GlcNAc) units and serves as a structural polymer in crustacean shells, in the cell walls of fungi, and in the exoskeletons of arthropods. The biological roles of some chitinases are well established, such as in chitin cycling in the marine environment by Vibrio cholerae and other Vibrio spp. (1), in yeast cell division (2), and in cuticle turnover and nutrient digestion in arthropods (3). Many plant chitinases are induced upon microbial infection, and some have the potential to inhibit the growth of fungal pathogens, hence displaying a defensive role (4, 5). However, the biological functions of numerous chitinases are...
Substrate Specificities of Phylogenetically Diverse Chitinases

Overview of chitinases used in this study

| Protein | Gene | Species | Source |
|---------|------|---------|--------|
| StChiA | STM0018 | Salmonella Typhimurium LT2 | This study |
| StChiADCBM | SL0018&CBM (1936–2097) | Salmonella Typhimurium LT2 | This study |
| StChiA E223Q | STM0018, G697C | Salmonella Typhimurium LT2 | This study |
| StChiA E223QDCBM | STM0018, G697C, ΔCBM (1936–2097) | L. monocytogenes EGD-e | (21) |
| LmChiA | lmo1883 | L. monocytogenes EGD-e | This study |
| LmChiB | lmo105 | L. monocytogenes EGD-e | This study |
| LmChiBA5DCBM | lmo105 & ΔCBM (2130–2265) | P. pallidum PN500 | This study |
| PyrChitinase | EFA83839 | S. glossinidius str. morsitans | This study |
| SgChiA | SG1474 | S. griseus | Sigma |
| S. griseus chitinase | Unspecified | T. viride | Sigma |
| T. viride chitinasea | Unspecified | & | |

a The STM0018 and SL0018 genes have identical nucleotide sequence.

As virulence or symbiotic factors as much as, or rather than, target. We have also shown that human chitotriosidase cleaves LacNAc and LactiNAc (GalNAcβ1–4GlcNAc) from LacNAc-TMR and LactiNAC-TMR, respectively, with the latter having a turnover comparable to that of pNP-chitotrioside (17).

In this study we have cloned and characterized chitinases, which are phylogenetically related to the Salmonella enzyme StChiA, from the bacterial insect endosymbiont Sodalis glossinidius and the eukaryotic slime mold Polysphondylium pallidum (see Table 1 and Fig. 2). These three enzymes share the common characteristic that they lack a signal peptide but are still predicted to be secretory proteins (14).

For comparison we have included the phylogenetically unrelated chitinases LmChiA and LmChiB (18) from L. monocytogenes (see Table 1 and Fig. 2) and the commercially available chitinases from the actinobacteria Streptomyces griseus and the mold Trichoderma viride. The chitinases included in the present study all belong to the CAZY family 18 glycosidases, although it should be noted that the two commercial available chitinases from T. viride and S. griseus remain uncharacterized in this regard.

We observed that the Salmonella chitinase StChiA was able to bind to potential glycan targets in hosts by use of a carbohydrate microarray. In addition, we found that all enzymes surveyed (with one exception) hydrolyzed LactiNAc from fluorescently labeled conjugates, whereas several of the enzymes hydrolyzed LacNAc from analogous conjugates that are substrate analogs of glycans found in mammals.

EXPERIMENTAL PROCEDURES

Phylogenetic Analysis—The amino acid sequences of Salmonella Typhimurium SL1344 StChiA (SL0018), S. glossinidius str. morsitans SgChiA (SG1474), P. pallidum Ppchitinase (EFA83839), L. monocytogenes LmChiA (lmo1883), and LmChiB (lmo105) (Table 1) were from the GenBank™ database of the National Center for Biotechnology Information (www.ncbi.nlm.nih.gov/). Protein domain predictions including determination of the glycosyl hydrolase family 18 (GH_18) domains were obtained from the SMART website. Signal peptide predictions were performed using SignalP 4.0 from the Centre for Biological Analysis, Biocentrum, Technical University of Denmark. GH_18 domains of sequences representative of bacterial chitinases in addition to representatives of eukaryotic chitinases were included in the phylogenetic anal-
Production and Purification of Chitinases—The nucleotide sequences of the chitinase genes SG1474 of S. glossinidius, morsitans and EFA83839 of P. pallidum PN500 were codon-optimized for expression in Escherichia coli, and the genes were synthesized with an N-terminal His<sub>6</sub> tag flanked by Ncol/Xhol restriction sites (Epoch Biolabs, Missouri City, TX). The synthetic genes were cloned into pET15b expression vectors, which were transformed into chemically competent E. coli TOP10 cells (Invitrogen) for long term storage. For protein expression the vector carrying SG1474 was transformed into E. coli BL21(DE3) cells (Novagen, Darmstadt, Germany), whereas the vector carrying EFA83839 was transformed into E. coli BL21/TUNER™(DE3) cells (Novagen). 10 ng of pET-15b-SG1474 or EFA83839 were added to 50 µl of E. coli TOP10, E. coli BL21(DE3), or E. coli BL21/Tuner™(DE3) cells. The reaction mixture was incubated on ice for 30 min, heat-shocked at 42 °C for 30 s, and incubated on ice for an additional 2 min, then 200 µl of Luria broth (LB; Merck) was added. The reaction mixture was incubated at 37 °C for 60 min with shaking and plated on LB agar plates containing 100 µg/ml carbenicillin and incubated overnight at 37 °C. Transformants were re-streaked on carbenicillin-containing LB plates. The following day colonies were grown in LB and stored as glycerol stocks.

The L. monocytogenes EGDε chib gene (lmo0105) and a truncated version of chib (lmo1883ΔCBM) lacking the C-terminal carbohydrate binding domain (amino acids 715–755) were amplified through PCR with primers designed to exclude the respective signal peptide sequences but including a His<sub>6</sub> tag at the N terminus. The PCR products were cloned into the pET-46 Ek/LIC vector and introduced into E. coli BL21(DE3) according to the manufacturer’s instructions (Novagen pET-46 E/LIC vector kit). The absence of miss- and nonsense mutations was confirmed by sequencing (Macrogen, Seoul, Korea). The E. coli BL21(DE3) strain used for expression of the L. monocytogenes chitinase, LmChiA (lmo1883), was from a previous study (21). An E. coli BL21(DE3) strain carrying a Salmonella chitinase gene without the chitin-binding domain (SL0018ΔCBM) and used for expression of StChiAΔCBM was prepared in a previous study (14). Expression vectors for the production of Salmonella wild type chitinase, StChiA, and the inactive enzyme variants StChiA E223Q and StChiA E223QΔCBM were purchased from Epoch life sciences Inc. (Missouri City, TX). In brief, StChiA E223Q was obtained by substituting the active site glutamate with a glutamine residue by site-directed mutagenesis (E223Q, GAA→CAA), whereas ChiA E223QΔCBM was obtained by a further truncation from amino acid 646. The synthetic genes were cloned into Ndel/Xhol restriction sites of the pETb15 expression vector carrying a His-tag coding sequence. The resulting vectors were transformed into E. coli BL21(DE3) as previously described.

For protein purification, up to 1 liter of E. coli BL21(DE3) cells were grown at 30 °C to an A<sub>600</sub> of 0.4 before induction with 1 mM isopropyl 1-thio-β-D-galactopyranoside. After induction, growth was continued for 21 h at 30 °C. For improved recovery and purification of the Polysphondylium chitinase, PpChitinase, E. coli BL21/Tuner™(DE3) cells were grown at 37 °C to an A<sub>600</sub> of 0.6 and placed on ice to cool before induction with 0.3 mM isopropyl 1-thio-β-D-galactopyranoside. After induction, growth was continued for 21 h at 22 °C. The E. coli BL21(DE3) and/or E. coli BL21/Tuner cells were harvested by centrifugation and resuspended in 10 volumes of loading buffer, 20 mM MOPS, pH 7.2, containing 0.5 M NaCl and 5 mM imidazole. The cells were disrupted using a Constant Systems cell disruptor at 4 °C with a pressure of 1.36 kilobars. The lysate was centrifuged at 4 °C for 1.5 h at 48,000 × g, and the filtered supernatant was applied to a 1- or 5-mL HisTrap HP column (GE Healthcare) with a flow rate of 1 or 5 ml/min, respectively. For the Listeria enzymes, 1–2-mL columns of nickel-nitrilotriacetic acid-agarose (Qiagen) were used with a flow rate of 1 ml/min. All columns were washed with 100 ml of loading buffer before being eluted with 30 ml of 100 mM MOPS, pH 7.8, 0.5 M NaCl, and 5 mM imidazole.

For the Sodalis chitinase SgChiA, and the Salmonella StChiAΔCBM, fractions containing enzyme were combined, the buffer was exchanged to loading buffer by dialysis, and the enzymes were rechromatographed on a 1-mL HisTrap column with an imidazole gradient from 5 to 250 mM imidazole to obtain ultrapure enzymes. For all enzymes, the fractions containing enzyme were dialyzed against 50 mM sodium phosphate buffer, pH 6.0, at 4 °C and concentrated in a Vivaspin (10,000 Da cutoff for LmChiA and 30,000-Da cutoff for other enzymes). The protein concentration of the sample was determined by the Bradford method using a commercial kit (Bio-Rad) with bovine γ-globulin as a protein reference standard. Alternatively, protein concentration was measured with Qubit 2.0 fluorometer (Invitrogen) according to the manufacturer’s protocol. Commercially available enzymes of S. griseus (Sigma) and T. viride (a mixture of two chitinases; Sigma) were dissolved according to manufacturer’s instructions and included in the study as reference enzymes.

For the enzymes from Salmonella, Sodalis, Polysphondylium, and Listeria, purified in this study, 12–73 mg of recombinant protein were obtained per liter of cell culture after chromatography on HisTrap columns. The purity of proteins after isolation assessed by SDS-PAGE was as found previously (14).

Glycan Array Scanning—Glycan arrays were fabricated as described previously (22, 23) and contained 317 carbohydrate ligands (supplemental Table 1). The chips were first treated for 30 min with blocking buffer (50 mM ethanolamid in 50 mM borate buffer, pH 8.0) and subsequently for 30 min with 0.1 M phosphate-buffered saline (PBS) (10 mM Na<sub>2</sub>HPO<sub>4</sub>, 10 mM NaH<sub>2</sub>PO<sub>4</sub>, 138 mM NaCl, and 2.7 mM KCl, pH 7.4) (Sigma) containing 0.1% Tween 20 (ICN, MP Biomedicals, Santa Ana, CA). 1 ml of 200 µg/ml enzyme (StChiA, SgChiA E223Q, and StChiA E223QΔCBM) diluted in PLI-P buffer (0.5 M NaCl, 3 mM KCl, 1.5 mM KH<sub>2</sub>PO<sub>4</sub>, 6.5 mM Na<sub>2</sub>HPO<sub>4</sub>, 1% BSA, 1% Triton-X-100, pH 7.4), pH 7.4, was added to the slide and incubated for 60 min at 37 °C. The slide was washed in 1×PBS (0.01 M Na<sub>2</sub>HPO<sub>4</sub>, 0.01 M NaH<sub>2</sub>PO<sub>4</sub>, 0.138 M NaCl, and 0.0027 M KCl, pH 7.4) (Sigma) containing 0.1% Tween 20 (ICN), then 1 ml of rabbit anti-ChiA polyclonal antibody (CovalAb UK Ltd, Cambridge, UK) diluted to 1/1000 in PLI-P buffer was added, and...
the slide was incubated for 60 min at 22 °C. The slide was washed with 1×PBS, then 1 ml of labeled secondary antibody (Cy5 goat anti-rabbit IgG (H+L); Invitrogen) diluted 1/500 in PLI-P buffer was added before incubation for 60 min at 22 °C. The slide was then washed with 1×PBS, spin-dried, and stored in the dark until analysis. Fluorescence intensities were detected using a ScanArray 5000 (PerkinElmer Life Sciences) confocal scanner, and image analyses were carried out by using ScanArray Express 3.0 and the fixed 70-μm-diameter rings method as well as Microsoft EXCEL software.

Examination of Enzyme Activity—A survey of the activity of chitinases was initially carried out with the pNP chitin pseudo-substrates: pNP-GlcNAc, pNP-(GlcNAc)_2, pNP-(GlcNAc)_3, and the cellulose pseudo-substrate 4-nitrophenyl-β-D-cellobioside whose structures are shown in Fig. 1. In the survey, 5 μl of enzyme (40–2000 μg/ml) diluted in 50 mM sodium phosphate buffer, pH 6.0, and 45 μl of 1.8 mM substrate dissolved in 50 mM sodium phosphate buffer, pH 6.0, were incubated at 30 °C. To limit the conversion of substrates to a maximum of 15–20%, the following enzyme concentrations were used: SgChiA (400 μg/ml), StChiAΔCBM (200 μg/ml), PpChitinase (100 μg/ml), LmChiA (40 μg/ml), LmChiB (200 μg/ml), LmChiBΔCBM (200 μg/ml), T. viride mixture (67 μg/ml) and S. griseus (400 μg/ml). 50-μl samples were removed after 30 min, and the reaction was quenched by adding 250 μl of 0.4 M sodium carbonate (Sigma S2127). Absorbance at 405 nm was measured in a plate reader and corrected for absorption using a control sample with added sodium phosphate buffer, pH 6.0, instead of enzyme. After the initial screening, pNP-(GlcNAc)_2 absorption values were converted into concentrations by the use of a p-nitrophenol (Sigma) standard curve. Conversion rates were normalized against protein concentration and are listed as pmol/min/mg of protein.

Kinetic parameters for pNP-(GlcNAc)_2 were determined for SgChiA (23 μg/ml) and PpChitinase (20 μg/ml) by measuring initial rates of reaction at eight different substrate concentrations ranging from 0.007 to 1.8 mM during 25–120 min of incubation at 30 °C. K_m and V_max were calculated using the software GraphPad Prism 4.0 (GraphPad Software), and k_cat was estimated by dividing V_max by enzyme concentration.

The activity of chitinases toward hyaluronic acid (Sigma, H5388) was tested in a standard enzymatic assay of hyaluronidase (Sigma) using hyaluronidase (bovine testes, Sigma H3506)
as a positive control. Samples were prepared according to the manufacturer’s instructions and incubated at 37 °C for up to 40 h, and absorbance was read as % transmittance (%) at 600 nm.

Fluorescently labeled model substrates had a TMR tag covalently linked to carbohydrates via a hydrophobic linker ((carbohydrate)β-O-(CH₂)₃CONH(CH₂)₂NHzCO-TMR) (24, 25). The structures are shown in Fig. 1. Initial screening was carried out by TLC analysis where 1 µl of GlcNAc-TMR (1 mM), LacNAc-TMR (20 mM), LacdiNAc-TMR (20 mM), or Type I-TMR (Galβ1–3GlcNAc-TMR, 1 mM) were incubated with 5 µl of enzyme at ambient temperature for 2–24 h. Reaction progress was monitored by removing 1-µl aliquots for thin-layer chromatography on silica gel plates developed with CHCl₃/MeOH/H₂O (65/35/5) as described previously (17). The TMR-labeled compounds, which are brightly colored red/purple, can be visualized by eye. The *T. viride* chitinase mixture was used as a positive control for monitoring GlcNAc-TMR and LacdiNAc-TMR hydrolysis.

After screening by TLC, conversion rates of LacNAc-TMR and LacdiNAc-TMR for all chitinases were determined by monitoring product formation with 5 mM substrate at 30 °C by capillary electrophoresis. The reaction volume was 10 µl of CE running buffer (50 mM sodium phosphate buffer, pH 6.0, and enzyme concentration of substrate ranging from 0.080 to 10.5 mM at fixed enzyme concentrations by monitoring product formation with 8 different enzymes by screening an Argos 250B fluorescence detector (Flux Instruments, Basel, Switzerland) equipped with an excitation filter of 546.1/10 nm and an emission filter of 570 nm. All experiments were carried out at a normal polarity, *i.e.* inlet anodic. Data were processed with the PrinCE 7.0 software.

Ultra-HPLC-mass spectrometry analysis was employed to screen extended substrates terminating in LacNAc including LacNAcβ1–6LacNAcβ3-TMR, LacNAcβ1–6Galβ1–3GalNAc-TMR (extended core 2), and LacNAcβ1–2-Man-TMR substrates (Fig. 1), which are only available in limited amounts (26, 27). Assays were carried out in 4 µl containing 50 mM sodium phosphate buffer, pH 6.0, 1 µg of each enzyme, and 5 pmol of TMR-substrate. After overnight incubation at room temperature, a 1-µl aliquot was removed, mixed with 300 µl of buffer (10 mM ammonium formate, pH 4.5 (22%), acetonitrile (88%) (v/v)) for analysis by ultra-HPLC-MS as previously described (17).

**Nuclear Magnetic Resonance—Substrates, either pNP-(GlcNAc)₂, (GlcNAc)₃, LacNAc-TMR, or LacdiNAc-TMR (0.4–1 mg), were dissolved in 0.7 ml of buffer, transferred to 5-ml nuclear magnetic resonance (NMR) tubes, and standard one-dimensional ¹H NMR spectra of substrates were acquired before and after the addition of 5–50 µl of undiluted *Sg*ChiA and *St*ChiAΔCBM (LacNAc-TMR only). NMR spectra were recorded at 15 or 25 °C on a Bruker Avance 800 instrument and analyzed as previously described (14). Deuterated buffer was prepared by lyophilizing 5 ml of 50 mM sodium phosphate buffer, pH 6.0, followed by the addition of 2 ml of D₂O to the dried solids, re-lyophilization, and suspension in 5 ml of D₂O. Spectra were recorded at 799.3 MHz using tetramethylsilane (8 = 0 ppm) as the internal standard with a 32 scan composite presaturation.

**RESULTS**

**Phylogenetic Analyses—**A phylogenetic analysis of selected family 18 glycosyl hydrolase catalytic domains revealed that they cluster roughly into three distinct groups (colored in *red*, *green*, and *blue*, respectively, in Fig. 2). The groups colored in *red* and *blue* fit into Cluster A according to the analysis of Karlsson and Stenlid (28). Catalytic domains in these two groups in general carry a chitin-insertion domain conferring a deep substrate binding cleft, which suggests exo-activity (29). The group colored in blue contains *St*ChiA, *Sg*ChiA, and *Pp*Chitinase from *Salmonella*, *Sodalis*, and *Polysphondylium*, respectively, and is characterized by a lack of signal peptides, yet all are predicted to be secretory proteins. On the other hand, the green group containing *Lm*ChiB from *Listeria* and also human chitinases in general carry signal peptides. The group colored in *red* (Fig. 2) containing *Lm*ChiA fit into group IV in cluster B according to Karlsson and Stenlid (28). The red group is characterized by the lack of a chitin-insertion domain, which confers a shallow substrate binding cleft suggesting endo-activity (29).

**Glycan Array Scanning—**The glycan array scanning was performed to screen for ligand binding by the full-length active *Salmonella St*ChiA enzyme as well as inactive variants with or without the chitin binding domain. Average background signal was below 1000 relative fluorescence units (RFU), and average signals above 3000 RFU were considered as positives. The glycan array scanning showed binding of the inactive *Salmonella* chitinases (*St*ChiA E223Q and *St*ChiA E223QΔCBM) to GlcNAc-containing glycans including the chitin oligosaccha-
rides chitopentaose and chitohexaose (glycan no. 493 and 503; Table 2, supplemental Table 1). The active enzyme (StChiA) did not bind to the latter two compounds, unlike the inactive variants, showing that hydrolytic activity interfered with glycan binding. The active enzyme also bound to a range of non-chitinous substrates with binding to five LacNAc-containing compounds (compounds No. 384, 385, 420, 498, 229; Table 2, supplemental Table 1) as well as TF (Thomsen-Friedenreich antigen Galβ1–3GalNAc) antigen and TFα antigen structures (glycan number 88 and 89, respectively; Table 2 and supplemental Table 1).
TABLE 3
Overview of the initial substrate activity screening of Salmonella, Sodalis, Polysphondylium and Listeria chitinases monitored by spectrophotometric pNP- or TLC assay for all chitinases toward the chitin pseudo-substrates and the TMR linker. The inactive enzymes only bound to some of these substrates (compound numbers 384, 385, 420, and 498) suggesting that changes in the active site also affected binding.

| Assay   | Substrate | StChiΔCBM | SqChiA | PpChitinase | LmChiA | LmChiB | LmChiBΔCBM | T. viride mixture | S. griseus chitinase |
|---------|-----------|-----------|--------|-------------|--------|--------|-----------|-------------------|-------------------|
| pNP     | pNP-GlcNac|           |        |             |        |        |           |                   |                   |
|         | pNP-(GlcNac)₂ |           |        |             |        |        |           |                   |                   |
|         | pNP-(GlcNac)₃ |           |        |             |        |        |           |                   |                   |
| TLC     | GlcNac-TMR |           |        |             |        |        |           |                   |                   |
|         | LacNac-TMR |           |        |             |        |        |           |                   |                   |
|         | LacdiNAc-TMR |           |        |             |        |        |           |                   |                   |
|         | Type I-TMR |           |        |             |        |        |           |                   |                   |
|         | Hyaluronic acid |           |        |             |        |        |           |                   |                   |

* pNP-GlcNac: 4-nitrophenyl-α-glucosaminide; pNP-(GlcNac)₂, 4-nitrophenyl-α,N,N',N''-diacetyl-β-D-chitosamide; pNP-(GlcNac)₃, 4-nitrophenyl-β-D-N,N',N''-triacetylchitotriose; pNP-cellobioside, 4-nitrophenyl-β-D-cellobioside; NT, not tested.

TABLE 4
Conversion rates for hydrolysis of LacdiNAc-TMR, LacNac-TMR, and pNP-(GlcNac)₂ by Salmonella, Sodalis, Polysphondylium, and Listeria chitinases.

| Enzyme                     | LacdiNAc-TMR | LacNac-TMR | pNP-(GlcNac)₂ |
|----------------------------|--------------|------------|--------------|
| S. griseus chitinase       | 8.3 ± 0.0    | 5.1 ± 0.2  | 320 ± 60     |
| Sodalis CBM                | 151 ± 3      | 29.0 ± 0.1 | 2460 ± 40    |
| PpChitinase                | 5.0 ± 0.3    | 0.0 ± 0.0  | 1570 ± 350   |
| LmChiA                     | 5.0 ± 0.1    | 0.6 ± 0.6  | 5660 ± 520   |
| LmChiB                     | 33 ± 13      | 0.1 ± 0.2  | 1750 ± 50    |
| LmChiABCBM                 | 47.1 ± 0.0   | 0.0 ± 0.1  | 1630 ± 80    |
| T. viride chitinase mixture| 1232 ± 50    | 0.2 ± 0.3  | 6700 ± 80    |
| S. griseus chitinase mixture| 0.9 ± 0.7 | 0.3 ± 0.3  | 1380 ± 40    |

* 5 mM substrate, 30 °C, 120 min; analysis by capillary electrophoresis.
+ 1.8 mM substrate, 30 °C, 30 min analysis by spectrophotometry.
LacdiNAc consists of a GalNAc and a GlcNAc unit, and these two units gave distinct anomeric signals in 1H NMR. These signals can be seen in Fig. 5, which shows the hydrolysis of LacdiNAc-TMR by SgChiA. From the bottom, the first spectrum (Fig. 5A) shows the substrate before adding enzyme with the peak at 4.18 ppm from H1 of GlcNAc and the peak at 4.33 ppm from H1 of GalNAc. The spectra B, C, and D show the formation of the product, LacdiNAc, at 1, 3.5, and 9 h, respectively, after the addition of the enzyme. The peak at 4.37 ppm is the signal from H1 of α-GalNAc when the reducing end is α-GlcNAc, and the peak at 4.38 ppm is the signal from H1 of β-GalNAc when the reducing end is β-GlcNAc. The signals at 4.55 ppm is the H1 of β-GlcNAc reducing end, and the signal at 5.19 ppm is the H1 of α-GlcNAc reducing end. Note that the α-anomer is formed later than the reducing end β-anomer thus confirming that the reaction occurs with retention of configuration as the reducing end β-anomer is the only product of enzyme-catalyzed steps.

pNP-chitobiose, pNP-(GlcNAc)2, and chitotriose (GlcNAc)3 were also shown to be substrates of SgChiA as monitored by NMR with release of β-disaccharide (GlcNAc)2 from pNP-(GlcNAc)2 and release of β-mono- and β-disaccharides from the trisaccharide (GlcNAc)3, and slow mutarotation to α-mono- and α-disaccharide (data not shown). These are analogous to the results previously reported for Salmonella StChiA (14).

**DISCUSSION**

We have investigated the affinity of a Salmonella chitinase toward a large range of substrates by use of a glycan microarray technique followed by investigations of conversion rates of

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**TABLE 5**

Kinetic constants for *Salmonella*, *Sodalis*, and *Polysphondylium* chitinases with pNP-(GlcNAc)2, LacNAc-TMR, and LacdiNAc-TMR

| Enzyme           | pNP-(GlcNAc)2 | LacNAc-TMR | LacdiNAc-TMR |
|------------------|---------------|------------|--------------|
|                  | K<sub>m</sub> | k<sub>cat</sub> | k<sub>cat</sub>/K<sub>m</sub> | K<sub>m</sub> | k<sub>cat</sub> | k<sub>cat</sub>/K<sub>m</sub> |
| StChiAΔCBM       | 0.73<sup>a</sup> | 0.7<sup>b</sup> | 0.95         | 2.8 ± 0.1 | 0.046 | 0.017 |
| SgChiA           | 0.29 ± 0.09   | 3.1        | 10.7         | 4.7 ± 0.4 | 0.015 | 0.003 |
| PpChitinase      | 1.1 ± 0.4     | 10.3       | 9.6          | 0.99 ± 0.16 | 0.055 | 0.09 |

<sup>a</sup> Monitored by capillary electrophoresis.

<sup>b</sup> Not tested in this study. Data are from Larsen et al. (14).
Substrate Specificities of Phylogenetically Diverse Chitinases

A

![Graphs showing enzyme activity and substrate specificities](image)

B

![Diagram illustrating enzyme structure](image)

C

| Enzyme      | Composition of TMR Compounds (%) | Hydrolytic Conversion (%) |
|-------------|----------------------------------|---------------------------|
| StChiAΔCBM  | (a) 38  (b) 62  (c) 0            | 62                        |
| SgChiA      | 2      65      33            | 98                        |
| LmChiA      | 100    0       0             | n.d.                      |
| LmChiB      | 94     3       3             | 6                         |
selected substrates by several chitinases and, finally, by screening a small selection of potential biological substrates.

The glycan array scanning highlighted a specific affinity of the full-length *Salmonella* chitinase, *St* ChiA, toward various GlcNAc-containing glycans. As expected, chitin-like substrates such as chitopentaose and chitohexaose were targeted by this chitinase. Some GlcNAc-containing glycans of mammalian-like LacNAc structures were also targets (Table 2). LacNAc structures have been identified as terminating glycans of glycoproteins and glycolipids on vertebrate cells and in the human glycome (30, 31).

The inclusion of the inactive *Salmonella* variants *St* ChiA E223Q and *St* ChiA E223Q/H9004 CBM in the study ensured that a reliable overview of binding affinities was obtained.

Detailed analyses of hydrolysis of the most promising of these mammalian and/or insect-like substrates were examined by use of a wider range of bacterial and eukaryotic chitinases. The chitinases were selected from a broad phylogenetic range and included the related (Fig. 2) enzymes from the Enterobacteri-aceae *Salmonella* Typhimurium and *S. glossinidius* as well as the slime mold *P. pallidum* and the unrelated chitinases from the Firmicute *L. monocytogenes*. Some of the enzymes (the *Listeria Lm* ChiB and *Sodalis Sg* ChiA) encode additional domains annotated as chitin binding domains, whereas others contain only the catalytic domain (*Lm* ChiA, *St* ChiA/H9004 CBM, *Polysphondylium Pp* Chitinase; Fig. 2). Additional studies are needed to clearly distinguish the respective roles of the family 18 glycosyl hydrolase domain and additional domains. The observation of marginal differences in hydrolytic activities for *Lm* ChiB and *Lm* ChiB/H9004 CBM without the chitin binding domain (Tables 3 and 4) suggest that there is a minimal effect of the chitin binding domain on the hydrolytic activities under the conditions tested.

The enzymatic analyses were performed using the fluorescently labeled model substrates LacdiNAc-TMR and LacNAc-TMR. A general activity toward LacdiNAc-TMR was observed.

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**FIGURE 4.** Ultra-HPLC-mass spectrometry analyses of LacNAc-TMR before and after reaction with four different chitinases. A, chromatograms of the reaction mixtures were monitored with a fluorescence detector (λ<sub>ex</sub> = 540 nm; λ<sub>em</sub> = 580 nm), and each peak was identified by mass spectrometry. EU, emission units. B, structures of the substrate (a) and the products (LacNAc-TMR (b) and Linker-TMR (c)), corresponding to labels on the chromatograms. The yellow circle and the blue square indicate Gal and GlcNAc, respectively. C, hydrolytic reaction yields based on peak areas of the TMR products formed. For a presentation of the exact structures, please see Fig. 1 and supplemental Fig. 1.

---

**FIGURE 5.** Hydrolysis of LacdiNAc-TMR by *Sodalis* chitinase (SgChiA) monitored by one-dimensional proton NMR showing only the anomeric region. A, substrate before the addition of enzyme. The signal at 4.18 ppm is H1 of GlcNAc and 4.33 ppm H1 of GalNAc. B–D show LacdiNAc formed at 1 h (B), 3.5 h (C), and 9 h (D) after the addition of enzyme. The signal at 4.37 ppm is H1 of β-GalNAc, when the reducing end is β-GlcNAc. The signal at 4.38 ppm is H1 of β-GalNAc when the reducing end is α. The signal at 4.55 ppm is β-GlcNAc reducing end, and 5.19 ppm is α-GlcNAc reducing end.
for all of the chitinases examined, except S. g里斯us chitinase, with the release of LacdiNAc from LacdiNAc-TMR. This is consistent with LacdiNAc binding to the −2 and −1 subsites and the hydrophobic linker with TMR in the enzymes + subsites. The hydrolytic activity toward LacdiNAc-TMR was much lower, and only the SgChiA and StChiAΔCBM released LacNAc from LacdiNAc-TMR to any extent within the 2-h incubation time (Table 4). The increased activity toward LacdiNAc-TMR is attributed to the presence of two N-acetyl groups, on the reducing end for substrate-assisted catalysis and on the non-reducing end for additional recognition by the respective chitinases. It is notable that the T. viride enzyme mixture also exhibited a high degree of hydrolytic activity toward LacdiNAc-TMR. Further analyses are needed to elucidate which T. viride chitinase(s) is responsible for this activity and to evaluate the potential biological role. The general activity of chitinases toward a LacdiNAc-terminating substrate is a novel observation only reported previously for the human chitotriosidase (17).

Hydrolysis of LacNAc model substrates is also not commonly reported. In addition to the previous studies of a Salmonella chitinase and the human chitotriosidase by the authors of the present paper (14, 17) only two studies have demonstrated such activity expressed by the chitinases in the bacterial species Amycolatopsis orientalis (32) and Bacillus circulans (33). The Amycolatopsis chitinase not only hydrolyzed chitobiose from pNP-(GlcNAc)2 but also released free oligosaccharides and p-nitrophenol from Galβ1,4GlcNAcβ1,3Galβ1,4GlcNAcβ-pNP GlcNAcβ1,3Galβ1,4GlcNAcβ-pNP, and LacNAc-pNP (Galβ1,4GlcNAcβ-pNP). LacNAc and LacNAc-pNP were not detected in the course of tetrasaccharide hydrolysis, consistent with pNP occupying the +1 subsite and the tetrasaccharide occupying the −4 to −1 subsites.

To demonstrate that the activity toward LacdiNAc-TMR and LacdiNAc-TMR was not a minor side activity of the chitinases, we examined the substrate specificities and Michaelis-Menten kinetic parameters for LacdiNAc-TMR, LacdiNAc-TMR, and pNP-(GlcNAc)2, for StChiAΔCBM, SgChiA, and PpChitinase. StChiAΔCBM and SgChiA showed similar trends in hydrolytic activity toward LacdiNAc-TMR and LacdiNAc-TMR, with an increased activity for LacdiNAc-TMR for both enzymes as seen by the 2–4 times higher $k_{cat}$ values (Table 5). When comparing $k_{cat}$ and $k_{cat}/K_m$ values for LacdiNAc- and LacdiNAc-TMR to the higher $k_{cat}$ and $k_{cat}/K_m$ values for the chitin pseudo-substrate pNP-(GlcNAc)2, the $k_{cat}$ value for the StChiAΔCBM enzyme with LacdiNAc-TMR was still 25% that of the value observed with pNP-(GlcNAc)2 (Table 5). Even higher $k_{cat}$ values toward LacdiNAc-TMR relative to pNP-(GlcNAc)2 have been observed for the human chitotriosidase (17). The rather low level of activity indicated by the low $k_{cat}$ values observed for the PpChitinase toward LacdiNAc-TMR (Table 5) should be interpreted with caution.

For SgChiA and StChiAΔCBM the LacdiNAc motif may be a potential biological target as this substrate is commonly found in the insect glycome (34). Salmonella has been associated with cockroaches, Chironomus midges and flies (35–38), and S. glossinidius is an endosymbiont of the tsetse fly (Glossina spp., Ref. 39).

The presence of chitinases in P. pallidum may provide an activity of general importance, such as degradation of yeast prey, as they are also found in another major cellular slime mold genus, Dictyostelium (Fig. 2); these two genera mainly belong to different groups within the Dictyostelia (40, 41).

To investigate whether the observed activities toward LacdiNAc-TMR and LacdiNAc-TMR are biologically relevant, we screened the Listeria, Salmonella, and Sodalis chitinases (LmChiA and LmChiB, StChiAΔCBM, and SgChiA, respectively) for activities toward extended substrates including LacdiNAcβ1–6LacdiNAcβ-TMR, LacdiNAcβ1–6(Galβ1–3)GalNAcα-TMR (extended core 2), and LacdiNAcβ1–2-Man-TMR (Fig. 1). Only LacdiNAcβ1–6LacdiNAcβ-TMR was a substrate. It is of interest to note that the two enzymes (StChiAΔCBM and SgChiA) that showed a high catalytic activity toward this substrate both were associated with the phylogenetic group colored in blue and belonging to cluster A (Fig. 2; Ref. 28). Further studies are, however, necessary to demonstrate that this group of chitinases indeed has an increased preference for this target.

The stepwise hydrolysis pattern for LacdiNAcβ1–6LacdiNAcβ-TMR is consistent with the terminal LacNAc occupying the −2 and −1 subsites and the inner LacdiNAc-TMR occupying the +1 and +2 subsites. The hydrolysis of LacdiNAcβ1–6LacdiNAcβ-TMR can be attributed to the flexibility of the β1,6 linkage. The product of hydrolysis is LacdiNAcβ-TMR, which in turn was further hydrolyzed by SgChiA and LmChiB enzymes. In this case the linker occupies the + subsites. The product distribution profiles indicate that the tetrasaccharide is hydrolyzed twice as rapidly as LacdiNAc-TMR by the SgChiA, whereas they are equally good substrates for LmChiB. The inability of StChiAΔCBM to further hydrolyze LacdiNAcβ-TMR might be due to the shorter linker to TMR in this compound (I(CH2)2) compared with the linker to LacdiNAc-TMR used for kinetics (I(CH2)6) or to a greater affinity for tetrasaccharide such that it precludes binding and hydrolysis of LacdiNAcβ-TMR. This highlights caution in the interpretation of kinetic results when non-natural leaving groups such as fluorescent linkers or the widely used pNP are used. The hydrolysis patterns were distinct from those seen for Amycolatopsis chitinase, which bound all oligosaccharide substrates with pNP in the +1 subsite. These results are in agreement with what has recently been observed for the human chitotriosidase (17). It can be concluded that terminal LacNAc or LacdiNAc structure-linked β1,4 or β1,6 in oligosaccharides, glycoproteins, or glycolipids are potential substrates for several chitinases. Especially for the Salmonella StChiAΔCBM and Sodalis SgChiA, this activity was significant, whereas this was not for the case for the Listeria chitinases (Fig. 4). Interestingly, it has been proposed that GH_18 enzymes from the human gut bacterial symbiont Bacteroides thetaiotaomicron may target LacNAc repeats of O-glycan chains (42).

It can be suggested that for Salmonella StChiA and Sodalis SgChiA chitinases, glycans that contain either a LacNAc or LacdiNAc structure-linked β1,4 or β1,6 have a high catalytic activity toward this substrate both were associated with the phylogenetic group colored in blue and belonging to cluster A (Fig. 2; Ref. 28). Further studies are, however, necessary to demonstrate that this group of chitinases indeed has an increased preference for this target.

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Substrate Specificities of Phylogenetically Diverse Chitinases

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**Substrate Specificities of Phylogenetically Diverse Chitinases**
A Diverse Range of Bacterial and Eukaryotic Chitinases Hydrolyze the LacNAc (Galβ1-4GlcNAc) and LacdiNAc (GalNAcβ1-4GlcNAc) Motifs Found on Vertebrate and Insect Cells.

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Supplementary Table 1. Layout of glycan structures of glycan array.

| # in the print & plate layouts | Nr. | Spacered form of saccharide | Common name | Short name | Molecular weight | Molecular weight of OS with TFA | V, ul of 10 mM solution |
|-------------------------------|-----|-----------------------------|-------------|------------|------------------|-------------------------------|------------------------|
| Monosaccharides               |     |                             |             |            |                  |                               |                        |
| 1                             | 001 | Fucααα- sp3                 | L-α-Fuc     | aF         | 221.3            | 2                             |                        |
| 2                             | 002 | Galααα- sp3                 | α-Gal       | aA         | 237.3            | 2                             |                        |
| 3                             | 003 | Galβββ- sp3                 | β-Gal       | bA         | 237.3            | 351.3                         | 2                      |
| 4                             | 004 | GalNAcαα- sp0               | TnSer       | TnSer      | 308.3            | 2                             |                        |
| 5                             | 005 | GalNAcαα- sp3               | Tn           | Tn         | 278.3            | 392.3                         | 2                      |
| 6                             | 006 | GalNAcββ- sp3               | β-GalNAc    | bAN        | 278.3            | 392.3                         | 2                      |
| 7                             | 007 | Glcααα- sp3                 | α-Glc       | aG         | 237.3            | 351.3                         | 2                      |
| 8                             | 009 | Glcβββ- sp3                 | β-Glc       | bG         | 237.3            | 2                             |                        |
| 9                             | 010 | GlcNAcββ- sp3               | β-GlcNAc    | GN         | 278.3            | 392.3                         | 2                      |
| 10                            | 011 | GlcNAcββ- sp2               | β-GlcNAc    | GN-C2      | 264.3            | 378.3                         | 2                      |
| 11                            | 012 | GlcNAcββ- sp7               | β-GlcNAc    | GN-Ph      | 312.3            | 2                             |                        |
| 12                            | 013 | GlcNAcββ- sp8               | β-GlcNAc    | GN-PEG     | 484.6            | 598.6                         | 2                      |
| 13                            | 014 | Glc(NGc)ββ- sp4             | β-Glc(NGc)  | bGN(Gc)    | 293.3            | 2                             |                        |
| 14                            | 015 | HOCH₂(HOCH₂)₄CH₂NH₂          | aminoglucitol | glucitol | 181.2            | 2                             |                        |
| 15                            | 016 | Manααα- sp3                 | α-Man       | aM         | 237.3            | 351.3                         | 2                      |
| 16                            | 017 | Manααα- sp4                 | α-Man       | aM-Gly     | 236.2            | 2                             |                        |
| 17                            | 018 | Manβββ- sp4                 | β-Man       | bM         | 236.2            | 350.2                         | 2                      |
| 18                            | 019 | ManNAcββ- sp4               | β-ManNAc    | bMN        | 277.3            | 391.3                         | 2                      |
|   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| 19 | 020 | Rhα-sp3 | L-α-Rha | aR | 221.3 | 335.3 | 2 |
| 20 | 021 | GalNAcα-sp4 | β-GalNAc | bAN-Gly | 277.3 | 2 |
| 21 | 022 | GlcNAcβ-sp4 | β-GlcNAc | GN-Gly | 277.3 | 2 |
| 22 | 037 | 3-O-Su-Galβ-sp3 | 3-O-Su-β-Gal | bA3Su | 317.3 | 2 |
| 23 | 040 | 4-O-Su-GalNAcβ-sp4 | 4-O-Su-β-GalNAc | bAN4Su-Gly | 357.3 | 2 |
| 24 | 043 | 6-O-Su-GlcNAcβ-sp3 | 6-O-Su-β-GlcNAc | GN6Su | 358.4 | 2 |
| 25 | 044 | GlcAα-sp3 | α-glucuronic acid | aGU | 251.2 | 2 |
| 26 | 045 | GlcAβ-sp3 | β-glucuronic acid | bGU | 251.2 | 2 |
| 27 | 046 | 6-H₂PO₄Glcβ-sp4 | β-Glc6P | G6P | 316.2 | 2 |
| 28 | 047 | 6-H₂PO₄Manα-sp3 | α-Man6P | M6P | 339.2 (Na⁺) | 2 |
| 29 | 048 | Neu5Acα-sp3 | α-Neu5Ac | Sia | 366.4 | 2 |
| 30 | 049 | Neu5Acα-sp9 | α-Neu5AcBn | Sia-Bn | 471.5 | 2 |
| 31 | 050 | Neu5Acβ-sp3 | β-Neu5Ac | bSia | 366.4 | 2 |
| 32 | 051 | No formula available |   |   |   |   | 2 |
| 33 | 052 | Neu5Gcα-sp3 | α-Neu5Gc | aNeu5Gc | 382.4 | 2 |
| 34 | 053 | Neu5Gcβ-sp3 | β-Neu5Gc | bSia5Gc | 382.4 | 2 |
| 35 | 054 | 9-NAc-Neu5Acα-sp3 | 9-NAc-α-Neu5Ac | 9NAcSia | 407.4 | 2 |
| 36 | 055 | 3-O-Su-GlcNAcβ-sp3 | 3-O-Su-β-GlcNAc | GN3Su | 358.4 | 2 |

**Disaccharides**

|   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| 37 | 071 | Fucα1-2Galβ-sp3 | H₃di | Hdi | 383.4 | 497.4 | 2 |
| 38 | 072 | Fucα1-3GlcNAcβ-sp3 | Fa3GN | 424.5 | 538.5 | 2 |
| 39 | 073 | Fucα1-4GlcNAcβ-sp3 | Le | Le | 424.5 | 2 |
| 40 | 074 | No formula available |   |   |   |   | 2 |
| 41 | 075 | Galα1-2Galβ-sp3 | Aa2A | 399.4 | 2 |
| 42 | 076 | Galα1-3Galβ-sp3 | B₃di | Bdi | 399.4 | 513.4 | 2 |
| 43 | 077 | Galα1-3GlcNAcβ-sp3 | T₃di | Tab | 440.5 | 2 |
| 44 | 078 | Galα1-3GlcNAcα-sp3 | T₉αα | Taa | 440.5 | 554.5 | 2 |
| No. | Formula                   | Acceptor    | Mass        | Charge |
|-----|--------------------------|-------------|-------------|--------|
| 079 | No formula available     |             |             |        |
| 080 | Galα1-3GlcNAcβ-sp3       | Aa3GN       | 440.5       | 2      |
| 081 | Galα1-4GlcNAcβ-sp3       | α-LacNAc    | 440.5       | 554.5  |
| 082 | Galα1-4GlcNAcβ-sp8       | α-LacNAc    | 646.7       | 760.7  |
| 083 | Galβ1-6Glcβ-sp4          | melibiose   | 398.4       | 512.4  |
| 084 | Galβ1-2Galβ-sp3          | Ab2A        | 399.4       | 513.4  |
| 086 | Galβ1-3GlcNAcβ-sp2       | Leβ         | 426.4       |        |
| 087 | Galβ1-3Galβ-sp3          | Ab3A        | 399.4       | 513.4  |
| 088 | Galβ1-3GalNAcβ-sp3       | Tββ         | 440.5       |        |
| 089 | Galβ1-3GalNAcα-sp3       | TF          | 440.5       | 554.5  |
| 090 | No formula available     |             |             |        |
| 092 | Galβ1-4Glcβ-sp2          | Lac         | 385.4       | 499.4  |
| 093 | Galβ1-4Glcβ-sp4          | Lac         | 398.4       |        |
| 094 | Galβ1-4Galβ-sp4          | Ab4A        | 398.4       | 512.4  |
| 095 | Galβ1-4GlcNAcβ-OCH₂CH₂NH(Et) | LacNAc | 454.5       |        |
| 097 | Galβ1-4GlcNAcβ-sp3       | LacNAc      | 440.5       | 554.5  |
| 098 | Galβ1-4GlcNAcβ-sp5       | LacNAc      | 553.6       |        |
| 099 | Galβ1-4GlcNAcβ-sp8       | LacNAc      | 646.7       | 760.7  |
| 100 | Galβ1-6Galβ-sp4          | Ab6A        | 398.4       | 512.4  |
| 101 | GalNAcα1-3GalNAcβ-sp3    | Fs-2        | 481.5       | 595.5  |
| 102 | GalNAcα1-3Galβ-sp3       | Aβi         | 440.5       | 554.5  |
| 103 | GalNAcα1-3GalNAcα-sp3    | core 5      | 481.5       |        |
| 104 | GalNAcβ1-3Galβ-sp3       | ANb3A       | 440.5       | 554.5  |
| 106 | GalNAcβ1-4GlcNAcβ-sp3    | LacdiNAc    | 481.5       |        |
| 107 | GalNAcβ1-4GlcNAcβ-sp2    | LacdiNAc    | 467.5       | 581.5  |
| 109 | GalNAc(furanose)β1-4GlcNAcβ-sp2 | LacdiNAc | 467.5       |        |
| 110 | Glcα1-4Glcβ-sp3          | maltose     | 398.4       | 512.4  |
| 111 | Glcβ1-4Glcβ-sp4          | celllobiose | 398.4       |        |
| 112 | Glcβ1-6Glcβ-sp4          | gentiobiose | 398.4       |        |
|   | Formula | Repeat Unit | Mass 1  | Mass 2  | Repeat Count |
|---|---------|-------------|---------|---------|--------------|
| 71 | GlcNAcβ1-3GalNAcα-sp3 | core 3 | core3  | 481.5  | 2            |
| 72 | GlcNAcβ1-3Manβ-sp4   |       |        | 439.4  | 553.4        |
| 73 | GlcNAcβ1-4GlcNAcβ-Asn | chitobiose-Asn | Ch2-Asn | 538.5  | 2            |
| 74 | GlcNAcβ1-4GlcNAcβ-sp4 | chitobiose | Ch2-Gly | 480.5  | 594.5        |
| 75 | GlcNAcβ1-6GalNAcα-sp3 | core 6 | core6  | 481.5  | 595.5        |
| 76 | Manα1-2Manβ-sp4      |       |        | 398.4  | 512.4        |
| 77 | Manα1-3Manβ-sp4      |       |        | 398.4  | 512.4        |
| 78 | Manα1-4Manβ-sp4      |       |        | 398.4  | 512.4        |
| 79 | Manα1-6Manβ-sp4      |       |        | 398.4  | 512.4        |
| 80 | No formula available |       |        |        |              |
| 81 | Manα1-2Manα-sp4      |       |        | 398.4  | 512.4        |
| 82 | (6-Bn-Galβ1-4)GlcNAcβ-sp2 |       |        | 6'Bn-LacNAc | 6'Bn-LN | 516.6  | 2        |
| 83 | (6-Bn-Galα1-4(6-Bn)GlcNAcβ-sp |       |        | Bn2-α-LacNAc | Bn2-aLN | 606.7  | 2        |
| 84 | Galβ1-4Glcβ-sp4-Phe   | Lac    | Lac-Phe | 545.6  | 2            |
| 85 | Galβ1-4Glcβ-sp4-Trp    | Lac    | Lac-Trp | 584.6  | 2            |
| 86 | Fucα1-2(3-O-Su)Galβ-sp3 | 3-O-Su-Hdi | Hdi3Su | 463.5  | 2            |
| 87 | Galβ1-3(6-O-Su)GlcNAcβ-sp2 | 6-O-Su-Leα | LeC6Su-C2 | 506.5  | 2            |
| 88 | Galβ1-3(6-O-Su)GlcNAcβ-sp3 | 6-O-Su-Leα | LeC6Su | 520.5  | 2            |
| 89 | Galβ1-4(6-O-Su)GlcNAcβ-sp2 | 6-O-Su-Lac | Lac6Su | 465.4  | 2            |
| 90 | Galβ1-4(6-O-Su)GlcNAcβ-sp3 | 6-O-Su-LacNAc | LN6Su | 520.5  | 2            |
| 91 | GalNAcβ1-4(6-O-Su)GlcNAcβ-sp2 | 6-O-Su-LacNAc | LacdiNAc6Su | 547.5  | 2            |
| 92 | GalNAcβ1-4(6-O-Su)GlcNAcβ-sp2 | 6-O-Su-LacNAc | LacdiNAc6Su | 547.5  | 2            |
| 93 | 3-O-Su-Galβ1-3GalNAcα-sp3 | 3'-O-Su-TF | TF3'Su | 520.5  | 2            |
| 94 | 6-O-Su-Galβ1-3GalNAcα-sp3 | 6'-O-Su-TF | TF6'Su | 520.5  | 2            |
| 95 | 3-O-Su-Galβ1-4Glcβ-sp2 | SM3    | Lac3'Su | 465.3  | 2            |
| 96 | 6-O-Su-Galβ1-4Glcβ-sp2 | 6'-O-Su-Lac | Lac6'Su | 465.3  | 2            |
| 97 | 3-O-Su-Galβ1-3GlcNAcβ-sp2 | 3'-O-Su-Leα | LeC3'Su-C2 | 506.5  | 2            |
| 98 | 3-O-Su-Galβ1-3GlcNAcβ-sp3 | 3'-O-Su-Leα | LeC3'Su | 520.5  | 2            |
|   | Formula                                      | Mass (Da) | Charge |
|---|----------------------------------------------|-----------|--------|
| 95 | 3-O-Su-Galβ1-4GlcNAcβ-sp2                   | 506.5     | 2      |
| 96 | 3-O-Su-Galβ1-4GlcNAcβ-sp3                   | 520.5     | 2      |
| 97 | 4-O-Su-Galβ1-4GlcNAcβ-sp2                   | 506.5     | 2      |
| 98 | 4-O-Su-Galβ1-4GlcNAcβ-sp3                   | 520.5     | 2      |
| 99 | 6-O-Su-Galβ1-3GlcNAcβ-sp2                   | 506.5     | 2      |
| 100| 6-O-Su-Galβ1-3GlcNAcβ-sp3                   | 520.5     | 2      |
| 101| 6-O-Su-Galβ1-4GlcNAcβ-sp2                   | 506.5     | 2      |
| 102| 6-O-Su-Galβ1-4GlcNAcβ-sp3                   | 520.5     | 2      |
| 103| GlcAβ1-3GlcNAcβ-sp3                         | 454.3     | 2      |
| 104| GlcAβ1-6GlcNAcβ-sp3                         | 413.4     | 2      |
| 105| GlcNAcβ1-4-[HOOC(CH₂)CH]-3-O-GlcNAcβ-sp4    | 552.5     | 2      |
| 106| GMDP-Lys                                     | 823.9     | 2      |
| 107| Neu5Acα-2-3Galβ-sp3                         | 528.5     | 2      |
| 108| Neu5Acβ-2-6GalNAcα-sp3                      | 528.5     | 2      |
| 109| Neu5Acα-2-3GalNAcα-β-sp3                    | 569.6     | 2      |
| 110| Neu5Acβ-2-6GalNAcα-β-sp3                    | 569.6     | 2      |
| 111| Neu5Gccβ-2-6GalNAcα-β-sp3                   | 585.6     | 2      |
| 112| Neu5Gcβ-2-6GalNAcα-β-sp3                    | 585.6     | 2      |
| 113| 3-O-Su-Galβ1-4(6-O-Su)Glcβ-sp2              | 567.5 (Na⁺) | 2  |
| 114| 3-O-Su-Galβ1-4(6-O-Su)GlcNAcβ-sp3           | 622.6 (Na⁺) | 2  |
| 115| 6-O-Su-Galβ1-3(6-O-Su)GlcNAcβ-sp2           | 608.5 (Na⁺) | 2  |
| 116| 6-O-Su-Galβ1-4(6-O-Su)GlcNAcβ-sp2           | 608.5 (Na⁺) | 2  |
| 117| No formula available                         |           |        |
| 118| 3,6-O-Su₂-Galβ1-4GlcNAcβ-sp2                | 608.5 (Na⁺) | 2  |
| 119| 4,6-O-Su₂-Galβ1-4GlcNAcβ-sp2                | 608.5 (Na⁺) | 2  |
| 120| 4,6-O-Su₂-Galβ1-4GlcNAcβ-sp3                | 622.6 (Na⁺) | 2  |
| No. | Formula | Annot. | Mass | Charge |
|-----|---------|--------|------|--------|
| 186 | Neu5Acα2-8Neu5Acα2-sp3 | (Sia)$_2$ | (Sia)$_2$ | 679.6 (Na$^+$) |
| 187 | No formula available | | | |
| 188 | No formula available | | | |
| 189 | 3,6-O-Su$_2$-Galβ1-4(6-O-Su)GlcNAcβ-sp2 | 3',6,6'-tri-O-Su-LacNAc | LN3'66'Su3 | 710.6 (2Na$^+$) |
| 190 | Galβ1-4-(6-P)GlcNAcβ-sp2 | 6P-LacNAc | LN6P | 506.4 |
| 191 | 6-P-Galβ1-4GlcNAcβ-sp2 | 6'-P-LacNAc | LN6'P | 506.4 |
| 192 | GalNAcβ1-4(6-O-Su)GlcNAcβ-sp3 | 6-O-Su-LacdiNAc | LacdiNAc6Su | 561.5 |
| 193 | 3-O-Su-GalNAcβ1-4GlcNAcβ-sp3 | 3'-O-Su-LacdiNAc | LacdiNAc3'Su | 561.5 |
| 194 | 6-O-Su-GalNAcβ1-4GlcNAcβ-sp3 | 6'-O-Su-LacdiNAc | LacdiNAc6'Su | 561.5 |
| 195 | 6-O-Su-GalNAcβ1-4-(3-O-Ac)GlcNAcβ-sp3 | 6'-Su-3-O-Ac-LacdiNAc | 3Ac-LacdiNAc6'Su | 603.5 |
| 196 | 3-O-Su-GalNAcβ1-4(3-O-Su)-GlcNAcβ-sp3 | 3,3'-O-Su$_2$-LacdiNAc | LacdiNAc3,3'Su2 | 663.5(Na$^+$) |
| 197 | 3,6-O-Su$_2$-GalNAcβ1-4-GlcNAcβ-sp3 | 3',6'-Su$_2$-LacdiNAc | LacdiNAc3',6'Su2 | 663.5(Na$^+$) |
| 198 | 4,6-O-Su$_2$-GalNAcβ1-4GlcNAcβ-sp3 | 4',6'-O-Su$_2$-LacdiNAc | LacdiNAc4',6'Su2 | 663.5(Na$^+$) |
| 199 | 4,6-O-Su$_2$-GalNAcβ1-4-(3-O-Ac)GlcNAcβ-sp3 | 4',6'-Su$_2$-3-O-Ac-LacdiNAc | 3Ac-LacdiNAc4',6'Su2 | 705.5(Na$^+$) |
| 200 | 4-O-Su-GalNAcβ1-4GlcNAcβ-sp3 | 4'-O-Su-LacdiNAc | LacdiNAc4'Su | 561.5 |
| 201 | 3,4-O-Su$_2$-GalNAcβ1-4-GlcNAcβ-sp3 | 3',4'-Su$_2$-LacdiNAc | LacdiNAc3',4'Su2 | 663.5(Na$^+$) |

**Trisaccharides**

| No. | Formula | Annot. | Mass | Charge |
|-----|---------|--------|------|--------|
| 215 | Fucα1-2Galβ1-3GlcNAcβ-sp3 | Le$^d$, H (type 1) | LeD | 586.6 | 700.6 |
| 216 | Fucα1-2Galβ1-4GlcNAcβ-sp3 | H (type 2) | Htype2 | 586.6 | 700.6 |
| 217 | Fucα1-2Galβ1-3GalNAcα-sp3 | H (type 3) | Htype3 | 586.6 | 700.6 |
| 219 | Fucα1-2Galβ1-4Glcβ-sp4 | H (type 6) | Htype6 | 544.5 | 658.5 |
| No. | Formula | Description | M.W. | Ref. |
|-----|---------|-------------|------|------|
| 128 | Galα1-3Galβ1-4Glcβ-sp2 | | 547.5 | 2 |
| 129 | Galα1-3Galβ1-4Glcβ-sp4 | | 560.5 | 2 |
| 130 | Galα1-3Galβ1-4GlcNAcβ-sp3 | Galili (tri) | Galili3 | 602.6 | 2 |
| 131 | Galα1-4Galβ1-4Glcβ-sp2 | Pβ, Gb3, GbOse3 | Pk-C2 | 547.5 | 2 |
| 132 | Galα1-4Galβ1-4Glcβ-sp3 | Pβ, Gb3, GbOse3 | Pk | 561.5 | 2 |
| 133 | Galα1-4Galβ1-4GlcNAcβ-sp2 | | | 2 |
| 134 | Galα1-3 | Galβ-sp3 | Btri | 545.5 | 2 |
| 135 | Fucα1-2 | | | |
| 136 | Galα1-3 | Galβ-sp5 | Btri | 658.7 | 2 |
| 137 | Fucα1-2 | | Btri-C8 | 772.7 | 2 |
| 138 | Galβ1-2Galα1-4GlcNAcβ-sp4 | | 601.6 | 2 |
| 139 | Galβ1-3Galα1-4GlcNAcβ-sp4 | | 601.6 | 2 |
| 140 | Galβ1-4Galα1-3GalNAcα-sp3 | | 643.6 | 2 |
| 141 | Galβ1-4GlcNAcβ1-3GalNAcα-sp3 | | 757.6 | 2 |
| 142 | Galβ1-4GalNAcβ1-6GalNAcα-sp3 | | 643.6 | 2 |
| 143 | Fucα1-4 | | Leα | 586.6 | 2 |
| 144 | Galβ1-3 | GlcNAcβ-sp3 | Leα | 700.6 | 2 |
| 145 | | | | |
| 146 | | | | |
| 147 | | | | |
| 148 | | | | |
|      | Formula                                    | Name            | Mass1   | 2   |
|------|--------------------------------------------|-----------------|---------|----|
| 149  | GlcNAcα1-3Galβ1-4GlcNAcβ-sp2              | GNa3'LN-C2      | 629.6   | 2  |
| 150  | GlcNAcα1-3Galβ1-4GlcNAcβ-sp3              | GNa3'LN         | 643.6   | 2  |
| 151  | GlcNAcα1-6Galβ1-4GlcNAcβ-sp2              | GNa6'LN         | 629.6   | 2  |
| 152  | GlcNAcβ1-2Galβ1-3GalNAcα-sp3              | GN2'TF          | 643.6   | 757.6 | 2  |
| 153  | GlcNAcβ1-3Galβ1-3GalNAcα-sp3              | GN3'TF          | 643.6   | 2  |
| 154  | GlcNAcβ1-3Galβ1-4Glcβ-sp2                | GN3'Lac         | 588.6   | 2  |
| 155  | GlcNAcβ1-3Galβ1-4GlcNAcβ-sp2              | GN3'LN-C2       | 629.6   | 743.6 | 2  |
| 156  | GlcNAcβ1-3Galβ1-4GlcNAcβ-sp3              | GN3'LN         | 643.6   | 757.6 | 2  |
| 157  | GlcNAcβ1-4Galβ1-4GlcNAcβ-sp2              | GN4'LN         | 629.6   | 2  |
| 158  | GlcNAcβ1-4GlcNAcβ1-4GlcNAcβ-sp4           | chitotriose     | Ch3     | 683.6 | 2  |
| 159  | GlcNAcβ1-6Galβ1-4GlcNAcβ-sp2              | GN6'LN         | 629.6   | 2  |
| 160  | GlcNAcβ1-6GalNAcα-sp3                    | core 2         | core2   | 643.6 | 757.6 | 2  |
| 161  | GlcNAcβ1-6GalNAcα-sp3                    | core 4         | core4   | 684.7 | 798.7 | 2  |
| 162  | GlcNAcβ1-6GalNAcα-sp3                    |                |         |     |     |     |
| 163  | Manα1-6Manβ-sp4                           | Man3           | (Ma)3b  | 560.5 | 2  |
| 164  | Manα1-3Manβ-sp4                           |                |         |     |     |     |
| 165  | Galβ1-4GlcNAcβ-sp3                        |                | (Ab)2-3,4GN | 602.6 | 2  |
| 166  | 3-O-Su-Galβ1-3                            |                |         |     |     |     |
| 167  | Fucα1-4GlcNAcβ-sp3                        | Su-Le$^a$      | 3'SuLeA | 666.7 | 2  |
| 168  | 3-O-Su-Galβ1-4                            | Su-Le$^x$      | 3'SuLeX | 666.7 | 2  |
| 169  | Fucα1-3GlcNAcβ-sp3                        |                |         |     |     |     |
| 170  | Neu5Acα2-6GalNAcα-sp3                     | 6-SiaTF        | 6SiaTF  | 731.7 | 2  |
| 171  | Neu5Acα2-6Galβ1-3                         |                |         |     |     |     |
| 172  | Neu5Acα2-6Galβ1-3                         |                |         |     |     |     |
| 173  | Neu5Acα2-6GalNAcα-sp3                     | A3a(Sia)Tn     | 731.7   | 2  |
| 174  | Neu5Acα2-6GalNAcα-sp3                     |                |         |     |     |     |
| No. | Formula | Monosaccharides | Notes | MW | Ref. |
|-----|---------|----------------|-------|----|------|
| 291 | No formula available | | | | |
| 292 | Neu5Acα2-3Galβ1-3GalNAcα-sp3 | 3'Sia-TF Sia3'TF | 731.7 | 2 |
| 293 | Neu5Acαβ2-3Galβ1-4Glcβ-sp3 | 3'SL 3'SL | 690.7 | 2 |
| 294 | Neu5Acαβ2-3Galβ1-4Glcβ-sp4 | 3'SL 3'SL-Gly | 689.6 | 2 |
| 295 | Neu5Acαβ2-6Galβ1-4Glcβ-sp2 | 6'SL 6'SL-C2 | 676.6 | 2 |
| 296 | Neu5Acαβ2-6Galβ1-4Glcβ-sp4 | 6'SL 6'SL-Gly | 689.6 | 2 |
| 297 | Neu5Acβ2-6Galβ1-4Glcβ-sp2 | β-6'SL b6'SL | 676.6 | 2 |
| 298 | Neu5Acαβ2-3Galβ1-4GlcNAcβ-sp3 | 3'SLN 3'SLN | 731.7 | 2 |
| 299 | Neu5Acαβ2-3Galβ1-3GlcNAcβ-sp3 | 3'-SiaLe⁶ 3'SiaLeC | 731.7 | 2 |
| 300 | Neu5Acαβ2-6Galβ1-4GlcNAcβ-sp3 | 6'SLN 6'SLN | 731.7 | 2 |
| 301 | Neu5Acαβ2-6Galβ1-4GlcNAcβ-sp8 | 6'SLN 6'SLN-PEG | 938.0 | 2 |
| 302 | Neu5Acβ2-6Galβ1-4GlcNAcβ-sp3 | β6'SLN b6'SLN | 731.7 | 2 |
| 303 | Neu5Gcα2-6Galβ1-4GlcNAcβ-sp3 | 6'SLN(Gc) 6'SLN(Gc) | 747.7 | 2 |
| 304 | Neu5Gcβ2-6Galβ1-4GlcNAcβ-sp3 | β6'SLN(Gc) b6'SLN(Gc) | 747.7 | 2 |
| 305 | Neu5Acαβ2-3Galβ1-4GlcNAcβ-sp3 | 9-Nac-Neu5Acαβ2-6Galβ1-4GlcNAcβ-sp3 | 9'Nac-6'SLN | 788.8 | 2 |
| 306 | Neu5Acαβ2-3Galβ1-3GlcNAcα-sp2 | KDN-Le₃ | KDN-LeC | 676.63 | 2 |
| 307 | KDNα2-3Galβ1-3GlcNAcα-sp2 | KDN-Le₃ | KDN-LeC | 676.63 | 2 |
| 308 | KDNα2-3Galβ1-4GlcNAcα-sp2 | KDN-LacNAc | KDN-LN | 676.63 | 2 |
| 309 | Neu5Acαβ2-6GalNAcα-sp3 | Neu5Acαβ2-3 | Sia2-3,6Tn | 882.8 (Na⁺) | 2 |
| 310 | 3'SiaLacNAcβ-OCH₂CH₂CH₂NH-(3'SiaLacNAc-amide-sp3) | (3'SLN)² | 1445.4 | 2 |
| 311 | Neu5Acαβ2-3Galβ1-4GlcNAcβ-sp3 | 4''-Su-3'SLN | 3'SLN4''Su | 833.8 (Na⁺) | 2 |
| 312 | 4-O-Su-Neu5Acαβ2-3Galβ1-4GlcNAcβ-sp3 | 4''-Su-3'SLN | 3'SLN4''Su | 833.8 (Na⁺) | 2 |
| 313 | Neu5Acαβ2-3Galβ1-4-(6-O-Su)GlcNAcβ-sp3 | 9''-Su-3'SLN | 3'SLN9''Su | 833.8 (Na⁺) | 2 |
| 314 | Neu5Acαβ2-3Galβ1-3-(6-O-Su)GalNAcα-sp3 | 9''-Su-3'SLN | 3'SLN9''Su | 833.8 (Na⁺) | 2 |
| 315 | Neu5Acαβ2-6Galβ1-4-(6-O-Su)GlcNAcβ-sp3 | 6-Su-3'SLN | 3'SLN6Su | 833.8 (Na⁺) | 2 |
| 316 | Neu5Acαβ2-3Galβ1-4-(6-O-Su)GalNAcα-sp3 | 6-Su-3'SLN | 3'SLN6Su | 833.8 (Na⁺) | 2 |
| 317 | Neu5Acαβ2-6Galβ1-4-(6-O-Su)GalNAcα-sp3 | 6'Su-3'SLN | 3'SLN6Su | 833.8 (Na⁺) | 2 |
| 318 | Neu5Acαβ2-3Galβ1-3-(6-O-Su)GalNAcβ-sp3 | 6'Su-3'SLN | 3'SLN6Su | 833.8 (Na⁺) | 2 |
| 319 | Neu5Acαβ2-6Galβ1-4-(6-O-Su)GalNAcβ-sp3 | 6'Su-3'SLN | 3'SLN6Su | 833.8 (Na⁺) | 2 |
| 320 | Neu5Acαβ2-3Galβ1-4-(6-O-Su)GalNAcβ-sp3 | 6',4''-Su-3'SLN | 3'SLN6',4''Su2 | 935.8 (2Na⁺) | 2 |
| 321 | Neu5Acαβ2-6Galβ1-4-(6-O-Su)GalNAcβ-sp3 | 6',4''-Su-3'SLN | 3'SLN6',4''Su2 | 935.8 (2Na⁺) | 2 |
| 322 | Neu5Acαβ2-3Galβ1-3-(6-O-Su)GalNAcβ-sp3 | 6',4''-Su-3'SLN | 3'SLN6',4''Su2 | 935.8 (2Na⁺) | 2 |
| Tetrasaccharides | | | | |
|---|---|---|---|---|
| 196 | **359** | Galα1-3Galβ1-3GlcNAcβ-sp3Fucα1-2 | B (type 1) | Btype1 | 748.7 | 2 |
| 197 | **360** | Galα1-3Galβ1-4GlcNAcβ-sp3Fucα1-2 | B (type 2) | Btype2 | 748.7 | 2 |
| 198 | **361** | Galα1-3Galβ1-4GlcNAcβ-sp2Fucα1-2 | B (type 2) | Btype2-C2 | 734.7 | 2 |
| 199 | **362** | Galα1-3Galβ1-3GlcNAcα-sp3Fucα1-2 | B (type 3) | Btype3 | 748.7 | 2 |
| 200 | **363** | Galα1-3Galβ1-4GlcNAcβ-sp3Fucα1-2 | B (type 4) | Btype4 | 748.7 | 862.7 | 2 |
| 201 | **364** | Galα1-3Galβ1-4GlcNAcβ-sp3Fucα1-3 | αGalLe⁺ | aGalLeX | 748.7 | 2 |
| 202 | **365** | No formula available | | | | 2 |
| 203 | **366** | GalNAcα1-3Galβ1-3GlcNAcβ-sp3Fucα1-2 | A (type 1) | Atype1 | 789.8 | 903.8 | 2 |
| 204 | **367** | GalNAcα1-3Galβ1-4GlcNAcβ-sp2Fucα1-2 | A (type 2) | Atype2-C2 | 775.8 | 889.8 | 2 |
| 205 | **368** | GalNAcα1-3Galβ1-4GlcNAcβ-sp3Fucα1-2 | A (type 2) | Atype2 | 789.8 | 2 |
| 206 | **369** | No formula available | | | | 2 |
| 207 | **371** | Fucα1-4GlcNAcβ-sp3Fucα1-2Galβ1-3 | Leᵇ | LeB | 732.7 | 846.7 | 2 |
| 208 | **372** | Fucα1-2Galβ1-4GlcNAcβ-sp3Fucα1-3 | Leʸ | LeY | 732.7 | 846.7 | 2 |
| 209 | **373** | Galα1-3Galβ1-4GlcNAcβ1-3Galβ-sp3 | | | | | 2 |
| 312 | **374** | Galα1-4Galβ1-4GlcNAcβ-sp3 | | Aa2-3',4'LN | 764.7 | 2 |
| No. | Formula  | Neutral  | Monosaccharide  | Cluster  | M₁  | M₂  |
|-----|----------|----------|-----------------|----------|-----|-----|
| 375 | Galβ1-3GlcNAcβ1-3Galβ1-4Glcβ-sp4 | LNT | LNT | 763.7 | 877.7 | 2 |
| 376 | Galβ1-3GlcNAcβ1-3Galβ1-3GlcNAcβ-sp2 | LeCb3'LeC | 791.8 | 905.8 | 2 |
| 377 | Galβ1-3GlcNAcβ1-3Galβ1-4GlcNAcβ-sp2 | LeCb3'LN | 805.8 | 919.8 | 2 |
| 378 | Galβ1-3GlcNAcβ1-3Galβ1-4GlcNAcβ-sp3 | LeCb6'LN | 791.8 | 905.8 | 2 |
| 379 | Galβ1-3GlcNAcβ1-4Galβ1-4Glcβ-sp3 | Asialo-GM1 | aGM1 | 764.7 | 2 |
| 380 | Galβ1-3GlcNAcβ1-3Galβ1-4Glcβ-sp4 | LNNt | LNNt | 763.7 | 2 |
| 381 | Galβ1-3GlcNAcβ1-3Galβ1-4GlcNAcβ-sp2 | i| LNb3'LN-C2 | 791.7 | 2 |
| 382 | Galβ1-3GlcNAcβ1-3Galβ1-4GlcNAcβ-sp3 | i | LNb3'LN | 805.8 | 2 |
| 383 | Galβ1-3GlcNAcβ1-3Galβ1-4Glcβ-sp2 | LeCb6'LN | 791.7 | 2 |
| 384 | Galβ1-4GlcNAcβ1-3Galβ1-4GlcNAcβ-sp3 | i | LNb3'LN | 805.8 | 2 |
| 385 | Galβ1-4GlcNAcβ1-3Galβ1-4GlcNAcβ-sp3 | i | LNb6'LN | 791.7 | 2 |
| 386 | Galβ1-4GlcNAcβ1-3Galβ1-4GlcNAcβ-sp3 | LNb6'LN | 791.7 | 2 |
| 387 | Galβ1-4GlcNAcβ1-3Galβ1-4GlcNAcβ-sp3 | LNb6'LN | 791.7 | 2 |
| 388 | Galβ1-4GlcNAcβ1-6GalNAcα-sp3 | LNb6TF | 805.8 | 919.8 | 2 |
| 389 | GalNAcβ1-3Galα1-4Galβ1-4Glcβ-sp3 | Gb4, P | Gb4 | 764.7 | 2 |
| 390 | (Glcα1-4)β-sp4 | maltotetraose | (Ga4)4b | 722.7 | 836.7 | 2 |
| 391 | (Glcα1-6)β-sp4 | isomaltotetraose | (Ga6)4b | 722.7 | 2 |
| 392 | GlcNAcβ1-4Galβ1-4GlcNAcβ-sp2 | GN2-3',4'LN | 832.8 | 2 |
| 393 | GlcNAcβ1-6Galβ1-4GlcNAcβ-sp2 | Tk | Tk | 832.8 | 2 |
| 394 | GlcNAcβ1-4Galβ1-4GlcNAcβ-sp2 | GN2-3',4'LN | 832.8 | 2 |
| 395 | GlcNAcβ1-6Galβ1-4GlcNAcβ-sp2 | Tk | Tk | 832.8 | 2 |
| 396 | GalNAcβ1-4Galβ1-4Glcβ-sp4 | LeCb3'LN | 791.8 | 2 |
| 397 | Galβ1-3GlcN(Fmi)β1-3Galβ1-4GlcNAcβ-sp3 | LeC(Fm)b3'LN | 791.8 | 2 |
| 398 | 3-O-SuGalβ1-4GlcNAcβ1-3Galβ1-4GlcNAcβ-sp3 | (3'SuLN)3'LN | 907.8 | 2 |
| 399 | 4-O-SuGalβ1-4GlcNAcβ1-3Galβ1-4GlcNAcβ-sp3 | (4'SuLN)3'LN | 907.8 | 2 |
| 400 | GalNAcβ1-4 | GM2 | GM2 | 879.8 | 2 |
| 401 | Galβ1-4Glcβ-sp2 | Neu5Acα2-3 | GM2 | 879.8 | 2 |
|   | Formula | 2 | 3'SLNb3A | 893.9 | 4 | 2 |
|---|---------|---|----------|-------|---|---|
| 231 | Neu5Acα2-3Galβ1-4GlcNAcβ1-3Galβ-sp3 |  |  |  |  |  |
| 232 | Neu5Acα2-3Galβ1-4GlcNAcβ-sp3 | SiaLeα | SiaLeX | 877.9 |  |  |
| 233 | No formula available, SleXfuc-beta |  |  |  |  |  |
| 234 | Neu5Acα2-3Galβ1-3 | SiaLeα | SiaLeA | 877.9 |  |  |
| 235 | Neu5Acα2-3Galβ1-4 | SiaLeα | SiaLeA-Gly | 876.8 |  |  |
| 236 | Neu5Acα2-3Galβ1-4 | SiaLeX6Su | 979.9 (Na⁺) |  |  |  |
| 237 | Neu5Acα2-3(6-O-Su)Galβ1-4 | SiaLeX6'Su | 979.9 (Na⁺) |  |  |  |
| 238 | No formula available |  |  |  |  |  |
| 239 | No formula available |  |  |  |  |  |
| 240 | No formula available |  |  |  |  |  |
| 241 | Neu5Acα2-6 | Sia2-TF | Sia2-3',6TF | 1044.9 (Na⁺) |  |  |
| 242 | Neu5Acα2-8Neu5Acα2-3Galβ1-4Glcβ-sp4 | GD3 | GD3 | 1002.9 (Na⁺) |  |  |
| 243 | No formula available |  |  |  |  |  |
| 244 | No formula available |  |  |  |  |  |
|   | **Penta-nona saccharides** |  |  |  |  |  |
| 245 | Fucα1-2Galβ1-3GlcNAcβ1-3Galβ1-4Glcβ-sp4 | LNFP-I | Htype1Lac | 909.9 | 1023.9 | 2 |
| 246 | Fucα1-2Galβ1-3GlcNAcβ1-3Galβ1-4GlcNAcβ-sp2 | H(type 1) penta | Htype1LN | 937.91 |  |  |
| 247 | Galα1-3Galβ1-4GlcNAcβ1-3Galβ1-4Glcβ-sp4 | Galili (penta) | Galili5 | 925.8 | 1039.8 | 2 |
| 248 | Galα1-3 | BLeα | BLeY | 894.9 |  |  |
| 249 | No formula available |  |  |  |  |  |
| 250 | No formula available |  |  |  |  |  |
| No. | Formula                        | Nomenclature          | Mass (Da) | Repetitions |
|-----|--------------------------------|-----------------------|-----------|-------------|
| 251 | Galβ1-4GlcNAcβ1-6              | GalNAcα-sp3           | LN2-3,6Tn | 1008.9      | 2           |
| 252 | Galβ1-4GlcNAcβ1-3              |                       |           |             |             |
| 253 | GlcNAcβ1-6                     | Galβ1-4GlcNAc-sp2     | LN3'(GN6')LN | 994.9      | 2           |
| 254 | GlcNAcβ1-3                     | Galβ1-4GlcNAcβ-sp2    | LN6'(GN3')LN | 994.9      | 2           |
| 255 | (Glcα1-6)β-sp4                 | isomaltopentaose      | (Ga6)5b   | 884.8       | 998.8       | 2           |
| 256 | Manα1-6                        | Man5                  | (Ma)5b    | 966.9       |             |             |
| 257 | Fucα1-4                        |                       |           |             |             |
| 258 | Fucα1-2Galβ1-3                 |                       |           |             |             |
| 259 | Galβ1-4GlcNAcβ1-3              |                       |           |             |             |
| 260 | (Glcα1-6)β-sp4                 | maltohexaose          | (Ga6)6b   | 1046.9      | 1160.9      | 2           |
| 261 | (GlcNAcβ1-4)β-sp4              | chitohexaose          | Ch6       | 1293.3      |             |             |
| 262 | (A-GN-M)2-3,6-M-GN-GNβ-sp4     | 9-OS                  | 9-OS      | 1697.6      |             |             |
| 263 | (GN-M)2-3,6-M-GN-GNβ-sp4       | 7-OS                  | 7-OS      | 1373.3      |             |             |
| 264 | Neu5Acα2-3Galβ1-4GlcNAcβ1-3Galβ1-4GlcNAcβ-sp2 | 3'SLN-LacNAc 3'SLN-LN | 1083.02   |             |             |
| 265 | Neu5Acα2-3Galβ1-4              |                       | SiaLe'3Gal | 1040.0      |             |             |
| 266 | Neu5Acα2-6                     |                       | LSTb      | 1055.0      |             |             |
| 267 | No formula available (Neu5Acα2-3Galβ1)2-3,4-GlcNAc-sp3 | | | |             |             |
| Comp. | Structure | Short Name | Comp. | Structure | Short Name |
|-------|-----------|------------|-------|-----------|------------|
| 268   | GalNAcβ1-4Galβ1-4Glc-sp2 | GD2 | 269   | Neu5Acα2-8Neu5Acα2-3Galβ1-4Glc-sp2 | GT3 |
| 270   | GalNAcβ1-4Galβ1-4Glc-sp2 | GD2 |
|       | (Neu5Acα2-8)Neu5Acα2-3 | |
| 271   | 624 (GlcAβ1-4GlcNAcβ1-3)-NH₂-ol | HyalU-ol | 272   | (Sia2-6A-GN-M)₂-3,6-M-GN-GN-NH₂-ol | 11-OS-ol |
| 273   | (Sia2-6A-GN-M)₂-3,6-M-GN-GNβ-sp4 | 11-OS-ol |
| 317   | 900 H-(Gly)₂-NH₂ Gly6-amide, linear | Gly6 |
|       | comp 1 GlcNAcβ2Man5GlcNAcβ4GlcNAcβ4GlcNAcβ-sp6 | NGA2B |
|       | comp 2 Galβ1GlcNAcβ2Man5GlcNAcβ4GlcNAcβ4GlcNAcβ-sp6 | 4'-NA2B |
|       | comp 3 Neu5AcαGalβ1GlcNAcβ2Man5GlcNAcβ4GlcNAcβ4GlcNAcβ-sp6 | 3'-A2B |
|       | comp 4 Neu5AcαGalβ1GlcNAcβ2Man5GlcNAcβ4GlcNAcβ4GlcNAcβ-sp6 | 6'-A2B |
|       | comp 5 GlcNAcβ2Man5GlcNAcβ4GlcNAcβ4GlcNAcβ4GlcNAcβ4GlcNAcβ-sp6 | NGA3B(2-4) |
|       | comp 6 Galβ1GlcNAcβ2Man5GlcNAcβ4GlcNAcβ4GlcNAcβ4GlcNAcβ-sp6 | 4'-NA3B(2-4) |
|       | comp 7 Neu5AcαGalβ1GlcNAcβ2Man5GlcNAcβ4GlcNAcβ4GlcNAcβ4GlcNAcβ-sp6 | 3'-A3B(2-4) |
|       | comp 8 Neu5AcαGalβ1GlcNAcβ2Man5GlcNAcβ4GlcNAcβ4GlcNAcβ4GlcNAcβ-sp6 | 6'-A3B(2-4) |
|       | comp 9 GlcNAcβ2Man5GlcNAcβ4GlcNAcβ4GlcNAcβ4GlcNAcβ4GlcNAcβ-sp6 | NGA3B(2-6) |
|       | comp 10 Galβ1GlcNAcβ2Man5GlcNAcβ4GlcNAcβ4GlcNAcβ4GlcNAcβ-sp6 | 4'-NA3B(2-6) |
|       | comp 11 Neu5AcαGalβ1GlcNAcβ2Man5GlcNAcβ4GlcNAcβ4GlcNAcβ4GlcNAcβ-sp6 | 3'-A3B(2-6) |
|       | comp 12 Neu5AcαGalβ1GlcNAcβ2Man5GlcNAcβ4GlcNAcβ4GlcNAcβ4GlcNAcβ-sp6 | 6'-A3B(2-6) |
### Concentration of the controls

| Short name | Controls | Initial concentration | 1st conc. or dilution | 2nd conc. or dilution |
|------------|----------|-----------------------|-----------------------|-----------------------|
| StrCy3     | Streptavidin-Cy3 (Invitrogen) | 1 mg/ml | 50 ug/ml | 10 ug/ml |
| hIgG       | human IgG (Sigma) | 10 mg/ml | 500 ug/ml | 100 ug/ml |
| mIgG       | murine IgG (Sigma) | 1 mg/ml | 500 ug/ml | 100 ug/ml |
| FGF23      | 20-mer peptide-biotin (FGF23) (Blixt) | 10 mg/ml | 100 ug/ml | 20 ug/ml |
| iM-FITC    | 2-mer peptide-FITC (iM) (Blixt) | ? | 1 : 2 | 1 : 10 |

**LEGEND:**
- sp2 = C2 = -O(CH₂)₂NH₂
- sp3 = C3 = -O(CH₂)₃NH₂
- sp4 = Gly = -NHCOCH₂NH₂
- sp5 = C₈ = -O(CH₂)₈NH₂
- sp6 = Ox = -N(Me)O(CH₂)₃NH₂
- sp7 = Ph = -OC₆H₄-p-NH₂
- sp8 = PEG = -(OCH₂CH₂)₆NH₂
- sp9 = Bn = -OCH₂C₆H₄-p-NHCOCH₂NH₂

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Each vial contains 2 µl 10 mM oligosaccharide solution (20 nmol of the OS) in 20% (v/v) DMSO in H₂O for 200 µl of the 100 µM solution preparation

Comp # - compounds from Carlo Unvergazt (obtained in October 2009-11-28)
sp0 = other spacers: Asn, Ser, C2Et (see details in the column "Spacered form of saccharide")
Asn = asparagine, NH-CO-CH₂CH(COOH)NH₂
Ser = serine, -OCH₂CH(COOH)NH₂
C2Et = -O(CH₂)₂NHEt

A = Gal
AN = GalNAc
Ch = chito
F = L-Fuc
G = Glc
Gc = glycolyl
GN = GlcNAcβ
R = Rha
i = iso
Lac = lactose
LN = N-acetyllactosamine
M = Man
MN = ManNAc
Malt = maltose
OS = oligosaccharide
P = phosphate
S = Sia = Neu5Acα
Su = sulfate
Tn = GalNAcα
U = uronic acid
Supplementary Fig 1. Structures and molecular weights of LacNAcβ1,6-LacNAc-TMR, and products of chitinase hydrolysis.
A Diverse Range of Bacterial and Eukaryotic Chitinases Hydrolyzes the LacNAc (Galβ1–4GlcNAc) and LacdiNAc (GalNAcβ1–4GlcNAc) Motifs Found on Vertebrate and Insect Cells

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