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Galactooligosaccharides (GOS) Inhibit *Vibrio cholerae* Toxin Binding to Its GM1 Receptor

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It is widely reported that cholera toxin (Ctx) remains a significant cause of gastrointestinal disease globally, particularly in developing countries where access to clean drinking water is at a premium. Vaccines are prohibitively expensive and have shown only short-term protection. Consequently, there is scope for continued development of novel treatment strategies. One example is the use of galactooligosaccharides (GOS) as functional mimics for the cell-surface toxin receptor (GM1). In this study, GOS fractions were fractionated using cation exchange chromatography followed by structural characterization using a combination of hydrophilic interaction liquid chromatography (HILIC) and electrospray ionization mass spectrometry (ESI-MS) such that their molecular weight profiles were known. Each profile was correlated against biological activity measured using a competitive inhibitory GM1-linked ELISA. GOS fractions containing >5% hexasaccharides (DP_6) exhibited >90% binding, with EC_{50} values between 29.27 and 56.04 mg/mL. Inhibition by GOS DP_6 was dose dependent, with an EC_{50} value of 5.10 mg/mL (5.15 μM MW of 990 Da). In removing low molecular weight carbohydrates that do possess prebiotic, nutraceutical, and/or biological properties and concentrating GOS DP_5 and/or DP_6, Ctx antiadhesive activity per unit of (dry) weight was improved. This could be advantageous in the manufacture of pharmaceutical or nutraceutical formulations for the treatment or prevention of an acute or chronic disease associated with or caused by the adhesion and/or uptake of a Ctx or HLT.

KEYWORDS: Mass spectrometry; HILIC; galactooligosaccharide; antiadhesive, *Vibrio cholerae* toxin; glycomimetic

INTRODUCTION

There is a consensus that oligosaccharide-rich foods are beneficial to health. Some have functional effects, similar to soluble dietary fiber (*1, 2*), and are structurally related to naturally occurring cell surface glycoconjugates and immunomodulatory components (*3, 4*). For example, antiadhesive oligosaccharides that mimic epithelial cell surface receptors have been unequivocally proven in experiments with a variety of bacterial pathogens to prevent adherence, thereby halting subsequent pathogenesis of otherwise healthy epithelial surfaces and the onset of disease in vivo (*5*−*8*). For example, food-grade galactooligosaccharides (GOS) have already been linked to the beneficial effects of mammalian milk (*7*), and additionally they contain saccharide residues similar to those expressed on the binding site of the ganglioside GM1, the natural receptor for bacterial toxins including *Vibrio cholerae* toxin (Ctx) and *Escherichia coli* heat labile toxin (HLT) (*9*).

Receptor−ligand interactions are highly specific. One example includes the multivalent interaction between the oligosaccharide portion of GM1 (GM1-OS) presented on the cell surface and Ctx (*10, 11*). Multivalency ensures this interaction is very strong with a K_D value of 1.51×10^{-11} M (*12*). Studies concerning the contribution that each constituent saccharide residue makes toward Ctx binding suggest the importance of the terminal galactose and sialic acid (*13, 14*). Therefore, oligosaccharide-based antiadhesives containing similar saccharide residues should effectively compete for Ctx. This was exemplified in a recent study by Minke et al. (*15*), in which "receptor antagonists" were designed on the basis of galactose and tested for efficacy in a Ctx and HLT competitive ELISA. Results showed *m*-nitrophenyl α-galactoside to be the most efficient inhibitor with IC_{50} values over 100 times lower than that of galactose alone of 0.72 mM and 0.6 mM (*15*).

A prophylactic for Ctx is important considering *V. cholerae* has become resistant to the antibiotic polymyxin and antimicrobial resistance is increasing in many parts of the world (*16*). This multiple drug resistance has significant implications in the use of antibiotics as a treatment and control strategy and

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intensifies the urgency to develop alternative technologies. One solution is to target the toxin adhesion to host cells and consequently prevent infection.

In the case of Ctx, GOS are promising because they are a byproduct of the dairy industry, are inexpensive and nontoxic, are already approved for human consumption and contain galactosyl residues similar to those expressed on the binding site of GM1 (*17*). GOS are manufactured enzymatically using the purified D-lactose fraction of whey as a substrate (*17*). The β-galactosidase enzyme from *Aspergillus oryzae* has a transgalactosyl catalytic activity on lactose resulting in the formation of di- to octa-saccharides composed of 1−7 galactose units linked to a glucose molecule at the reducing end, that is, $(galactose)_n$glucose, where n is 1−7 (*18*). However, enzymatic synthesis produces GOS mixtures that are frequently impure, that is, the commercial syrup VivinalGOS contains 59% GOS w/w, with lactose, glucose, and galactose accounting for the remaining 41%. Among the GOS species present in VivinalGOS, disaccharides (DP_2) and trisaccharides (DP_3) are most abundant, representing approximately 19 and 23% w/w, respectively, based on the total dry weight of all VivinalGOS. Consequently, we purified the commercial VivinalGOS syrup to retain the nondigestible active compounds, followed by characterization of each fraction using HILIC coupled to ESI-MS so that the prevalence of each saccharide was known. Finally, we investigated the inhibition of Ctx binding to GM1-OS in the presence of GOS fractions using competitive ELISA. Resultant EC_{50} values were correlated with structural information from HILIC-ESI-MS experiments to elucidate inhibitory GOS structures.

EXPERIMENTAL PROCEDURES

Chemicals. All solutions were prepared using ultrapure Milli-Q water. Monosialoganglioside-GM1 isolated from bovine brain, Tween 20, bovine serum albumin (BSA), 3,3′,5,5′-tetramethylbenzidine (TMB), and sulfuric acid were all supplied by Sigma Aldrich (Gillingham, Dorset, U.K.). Phosphate-buffered saline (PBS) tablets containing 160 mM NaCl and 9 mM potassium phosphate were supplied by Oxoid Ltd. (Basingstoke, Hants, U.K.), and *V. cholerae* toxin B-subunit conjugated to horseradish peroxidase (Ctx−HRP) was supplied by Quadratech Ltd., Surrey, U.K. (manufactured by List Biologicals, Campbell, CA).

The commercial GOS mixture VivinalGOS had a typical composition of 75% w/w dry matter of which 59% w/w was GOS, 20% w/w was lactose (anhydrous), 19% w/w was glucose anhydrous, and 0.9% w/w was galactose (Friesland Foods Domo, Zwolle, The Netherlands). HPLC grade methanol and H_2O were purchased from Rathburns Chemical Co. (Peebleshire, Scotland), and ammonium acetate (NH_4OAc) was obtained from BDH (VWR International, Poole, U.K.). Fractionated GOS powders were produced using UBK-530 cation exchange resin (Mitsubishi Chemical Corp., Tokyo, Japan) in Na^+ form. 2-Aminobenzamide (2-AB) labeled glucose homopolymer (GHP) ladder was supplied by Ludger Ltd. (Abingdon, Oxfordshire, U.K.).

Determination of β-Galactosidase Enzyme Activity (Maxilact L5000). In the reaction, lactose is hydrolyzed to form glucose and galactose. These monosaccharides (DP_1) are retained with high affinity in cation exchange chromatography.

The activity of β-galactosidase from *Kluyveromyces lactis* (Maxilact L5000 from DSM Food Specialties, Delft, The Netherlands) was determined at 40 °C using 15 mM lactose in 0.2 M potassium phosphate buffer, pH 7.0, containing 2 mM $MgCl_2$ as the substrate. The enzyme was used without further purification and diluted using demineralized H_2O. Lactose substrate solution was maintained at 40 °C before enzyme addition to initiate the reaction. The enzyme was inactivated at 100 °C to prevent further hydrolysis and glucose concentration calculated using a glucose oxidase kit (Kit 510-A, Sigma Diagnostics, Gillingham, U.K.). A calibration curve was produced using standard glucose solutions (0−0.1 mg/mL), and absorbance was recorded spectrophotometrically at 450 nm (GENios spectrophotometer, Tecan U.K. Ltd., Theale, U.K.). One enzyme unit (U) was defined as 1 μmol of glucose released per milliliter per minute (1 μmol/mL/min) at 40 °C, pH 7.0.

Protein concentration in the enzyme preparation was determined using the bicinchoninic acid (BCA) assay (*19*). BSA was used as the model standard for calibration. Specific activity of the enzyme was then calculated from the fraction of the activity unit (U/mL) divided by protein concentration (mg/mL).

Hydrolysis of Lactose in VivinalGOS with β-Galactosidase; Maxilact L5000. Before cation exchange purification (IEX), 30% solution w/w of VivinalGOS was adjusted to pH 6.5 using 0.1 M sodium hydroxide. After heating to 40 °C, 0.9 g of Maxilact was added to the VivinalGOS and incubated for 4 h. Finally the solution was readjusted to pH 4.5 and heated for 10 min at 100 °C to deactivate the lactase, because GOS remain unaffected by high temperatures or harsh acidic conditions. Precipitated Maxilact was removed by centrifugation at 58000*g* for 60 min. The resultant lactose-free VivinalGOS solution was used in cation exchange chromatographic separations.

Preparative Scale Cation Exchange Chromatography (IEX) Purification of VivinalGOS. Modified from Tamura et al., 2 L of Na^+-form resin Unibead UBK-530 (Mitsubishi Chemical Industries Ltd.) was hydrated and fines were removed followed by treatment with 1 M KCl solution for 12 h in a stirring water bath at 20 °C to exchange the Na^+ ion with a K^+ ion (*20*). The resulting K^+ form resin was washed to remove excess salt before loading 1.8 L into a borosilicate glass column with dimensions of 5 × 100 cm and surrounded by an acrylic plastic thermostatic jacket (Pharmacia XK-50/100 column, GE Healthcare formerly Amersham Pharmacia Biotech, Buckinghamshire, U.K.). Eluent and analytes were injected using a pulsation-free three-piston pump module (model C-601, Buchi, Flawil, Switzerland) and post separation saccharides were detected by inline differential refractometry (Gilson model 132 RI detector, Anachem, Bedfordshire, U.K.). The column, samples, and eluent were heated to 55 °C to ensure equilibrium (model FE circulating water bath, Thermo Haake, Basingstoke, U.K.). Initially, the flow rate of demineralized water was set at 1 mL/min, increasing to 15 mL/min followed by loading with 150 mL of 30% w/w lactose-free GOS at 15 mL/min. The void volume was 675 mL, and GOS-rich eluent was collected every minute, in fractions, until no further saccharides were detected, followed by concentration using a rotary evaporator (Büchi Labortechnik AG) and freeze-drying. The resulting size-fractionated GOS powders were stored at −20 °C.

Hydrophilic Interaction Chromatography (HILIC). A ZIC-HILIC, PEEK column with dimensions of 150 × 2.1 mm and 5 μm particle size was selected because stationary phase "bleed" from the column has been shown to be particularly low (SeQuant AB, Umea, Sweden). Analysis parameters included the following: flow rate, 100 μL/min; 1 μL injection volume, 214 nm; UV detection. The eluent gradient comprised 95% MeOH NH_4OAc/5% H_2O → 50% MeOH NH_4OAc/50% H_2O in 40 min. A low water content of 5% was necessary to maintain suitable hydration and improve electrostatic interactions between stationary and mobile phases. The HPLC system consisted of an Agilent 1100 series capillary pump with an online degasser (Agilent, Stockport, U.K.), a UV detector, an autosampler, and a Dionex CS 14 cation exchange guard (4 mm × 50 mm).

HILIC Coupled to Electrospray Ionization Mass Spectrometry (HILIC-ESI-MS). GOS fractions were insoluble in >50% MeOH; therefore, iexGOS fractions were reconstituted in HPLC-grade 50:50 MeOH/H_2O containing 5 mM NH_4OAc. Samples were analyzed postchromatography using a microTOF MS equipped with an Apollo ion source (ESI, Bruker Daltonics, Bremen, Germany) and operated in the positive-ion mode. Other parameters included ESI voltage of 4500 V, capillary temperature of 200 °C, nebulizer gas pressure of 3.0 bar, and dry gas flow of 10 L/min.

Competitive GM1-Linked ELISA. These methods have been described fully (*21*) and validation of assays reported previously (*22*). Briefly, microtiter plates were incubated at room temperature overnight with 500 ng/mL GM1-OS dissolved per well in PBS (pH 7.2). Additional binding sites on the plate surface were blocked by incubating the wells with 2% w/v BSA−PBS solution overnight at room temperature. A dilution series of 1.56−100 mg/mL iexGOS fraction

was prepared in 0.1% w/v BSA−PBS and incubated with 5, 10, or 20 ng/mL Ctx−HRP, for 2 h at room temperature. A standard curve of 0, 0.97, 1.95, 3.90, 7.81, 15.62, 31.25, and 62.5 ng/mL Ctx−HRP was used on each plate to calculate unknown Ctx−HRP values. Following application to the plate, a further 2 h incubation period ensured immobilized GM1-OS could bind to any uninhibited Ctx−HRP. Finally, plates were treated with TMB solution for 15 min at room temperature, and the resultant chromogenic reaction was quenched with 2 M sulfuric acid before measurement spectrophotometrically at 450 nm (Genios, Tecan UK Ltd., Thatcham, U.K.). The GM1-OS ELISA was constrained by the linearity of the peroxidase substrate standard curve and the observation that half-maximal binding is estimated to be 15 ng/mL or 0.153 nM Ctx−HRP (*21*).

Each experiment was carried out in duplicate on three separate occasions, and all data were pooled. A control solution of 30 ng/mL Ctx−HRP was also used to measure the intra-assay coefficient of variation (CV). For analysis, Ctx−HRP concentration was transformed from nanograms per milliliter to percentage inhibition and iexGOS concentration normalized onto a log scale. Comparison between samples was made using a fitted four-parameter unweighted logistic curve (Prism v4 software, GraphPad Software, Inc., San Diego, CA), and ANOVA was calculated using Minitab version 14 (Mintab Ltd., Coventry, U.K.).

RESULTS

Determination of β-Galactosidase Activity. The β-galactosidase expressed a lactase activity of 5216 units/mL, where 1 unit (U) was defined as 1 μmol of glucose released per milliliter per minute at 40 °C and pH 7.0. Protein content in the enzyme was 78.1 mg/mL. Therefore, the enzyme expressed a specific activity of 67 U/mg. The Michaelis constant (K_M) and maximum rate (V_{max}) of the enzyme was determined by a Lineweaver−Burk plot. The K_M was 24.7 mM, and the V_{max} was 132 μmol of glucose/min/mg for lactose at 40 °C and pH 7.0.

Preliminary Separation of GHP Ladder by HILIC-ESI-MS. HILIC-ESI-MS feasibility was initially performed using a commercial GHP (*23*). **Figure 1** shows mass spectra obtained using positive ion polarity. Each saccharide was completely resolved on this column at 25 ± 0.1 °C with full elution within 60 min isocratically. The manufacturer recommends a flow rate of 100 μL/min to enhance interaction between contact region and stationary phase (*24*). It has been established that the reducing end is likely the contact region when anomeric separation is observed during HILIC (*25*). **Figure 1** shows complete separation between anomeric forms with decreasing detector response at each DP.

HILIC-ESI-MS Analysis of iexGOS Fractions. Preliminary experiments using higher column temperatures proved to be most effective at improving resolution with iexGOS fractions. At 60 ± 0.5 °C, a flow rate of 100 μL/min and gradient of 95% methanol, 5 mM ammonium acetate → 50% methanol, 5 mM ammonium acetate in water in 60 min partially resolved the composition of GOS fractions 1−15. Using the software integration features in Bruker Daltonics DataAnalysis v3.2 software, it was possible to integrate the area under each of the groups of saccharides identified as DP_3 followed by DP_4, DP_5, DP_6, and DP_7. **Figure 2** shows differences between DP_6 extracted ion chromatograms. By integrating, it was possible to calculate an abundance value for each DP in each iexGOS fraction. However, it has been shown that oligosaccharides do not ionize with the same efficiency in the source (*26*), and so expressing data in the form of relative abundance is common practice in mass spectrometry (*27*). For example, inequality in iexGOS ionization would make comparative analysis between fractions very difficult. Furthermore, if the total abundance of all saccharide adducts in each iexGOS fraction is added, percentages are calculated for each DP, and individual DP_6, DP_5, DP_4, DP_3 abundance are plotted relative to all ions formed, it is possible to compare fraction composition. **Figure 3** shows the relative abundance of DP_3−DP_6 against fraction number. Linear regression between fraction number and DP_6 abundance gives the closest r^2 of 0.956. DP_5 abundance is also inversely related to fraction number with $r^2 = 0.712$.

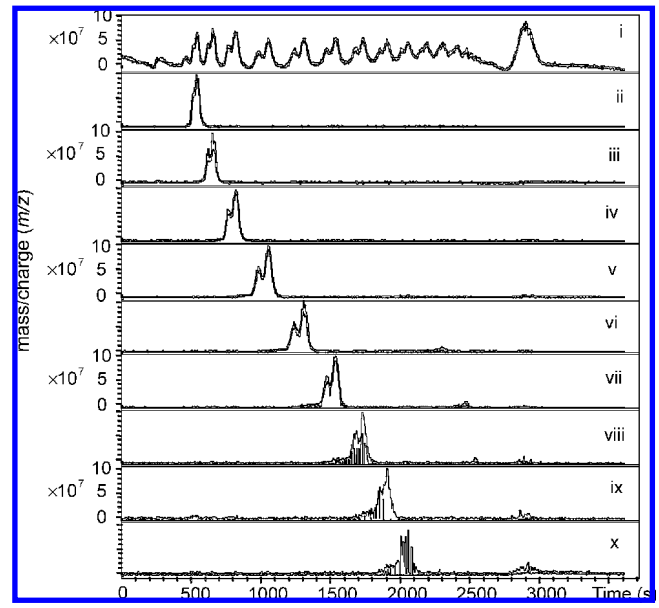

Figure 1. Separation of glucose homopolymer calibration standard. The abundance of individual ions was integrated by Data Analysis v3.3 software to produce chromatograms. Column: ZIC-HILIC 150 ± 2.1 mm held at 25 ± 0.5 °C. Eluent: 95% MeOH NH_4OAc/5% H_2O. Flow rate: 100 μL/min. Detection: ESI-MS. Injection: 1 μg/μL in mobile phase.

key	*m/z*	DP	cationization
i	100−2000	all	all
ii	365	2	Na^+
iii	527, 533	3	NH_4, Na^+
iv	684, 689	4	NH_4, Na^+
v	846, 851	5	NH_4, Na^+
vi	1008, 1013	6	NH_4, Na^+
vii	1175	7	Na^+
viii	1337	8	Na^+
ix	1499	9	Na^+
x	1661	10	Na^+

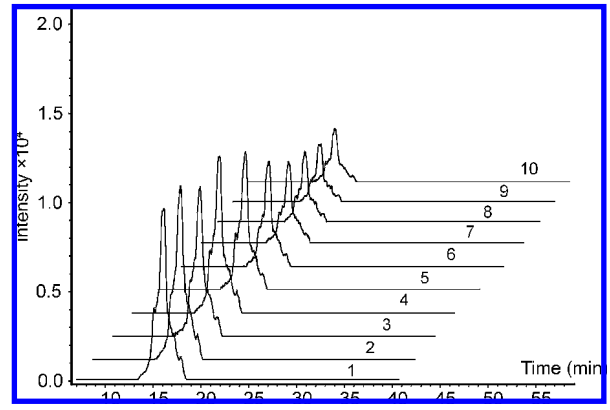

Figure 2. Extracted ion chromatograms of iexGOS fractions 1−10. The abundance of individual ions was integrated using Bruker Daltonics Data Analysis v3.3 software. Column: ZIC-HILIC 150 ± 2.1 mm held at 25 ± 0.5 °C. Eluent: 95% MeOH NH_4OAc/5% H_2O → 50% MeOH NH_4OAc/50% H_2O. Flow rate: 100 μL/min. Detection: ESI-MS. Injection: 1 μg/μL in mobile phase.

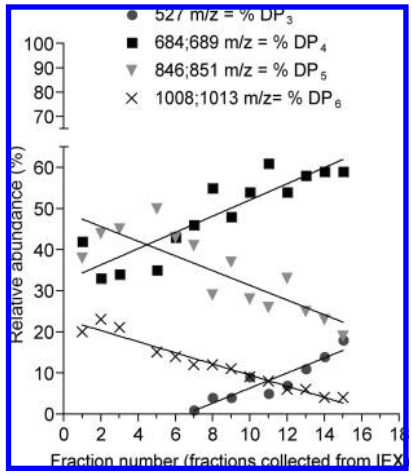

Figure 3. Extracted ion chromatograms of iexGOS fractions 1−15 integrated and expressed as relative abundance. These data show the change in composition from high concentrations of DP_6 and DP_5 containing fractions to high DP_4 and DP_3 containing fractions. Column: ZIC-HILIC 150 ± 2.1 mm held at 25 ± 0.5 °C. Eluent: 95% MeOH NH_4OAc/5% H_2O → 50% MeOH NH_4OAc/50% H_2O. Flow rate: 100 μL/min. Detection: ESI-MS. Injection: 1 μg/μL in mobile phase.

Inhibition of Ctx−HRP Binding to GM1 Using iexGOS. **Figure 4a** shows an interaction plot between the three concentrations of Ctx−HRP bound and the dose of iexGOS fraction 1. Sigmoidal dose−response curves and two-way ANOVA confirm that the increase in iexGOS fraction 1 concentration affects the Ctx−HRP inhibition ($P < 0.001$, $r^2 = 0.9843$, **Figure 4a**). Mean concentrations of Ctx−HRP bound without inhibitor (negative control) were 4.25, 8.31, and 21.90 ng/mL. A comparison between dose−response curves at each concentration of Ctx−HRP revealed that the EC_{50} values were not statistically different ($P = 0.9411$, $F = 0.061$), indicating that iexGOS fraction 1 inhibited Ctx−HRP with equal efficacy, irrespective of the amount of Ctx−HRP (**Figure 4b**). Furthermore, similar levels of inhibition were measured with fractions 1−9 with EC_{50} values varying minimally (**Table 1**) from 29.27 mg/mL (fraction 1) to 56.04 mg/mL (fraction 9). Ctx−HRP EC_{50} values could not be obtained for fractions 10−15 because either 100% inhibition was not observed or the sigmoidal dose−response curve fitted poorly; therefore, to show efficacy between fractions, the maximal inhibition with 100 mg/mL iexGOS was chosen rather than EC_{50}. One-way ANOVA between each iexGOS fraction at 100 mg/mL and 20 ng/mL (0.204 nM) Ctx−HRP inhibition revealed there was a statistical difference between fractions with $P < 0.001$ and $F = 9.60$ (**Figure 5**). Fractions 1−7 provide a minimum of 92% inhibition, whereas fractions 10−15 inhibit <66% Ctx−HRP with higher standard deviation between replicates (**Table 1** and **Figure 5**).

Correlation between the iexGOS Activity and Composition. The abundance of DP_6 was shown to correlate closely with fraction number (**Figure 3**); therefore, the mean of bound Ctx−HRP was plotted against relative abundance DP_6 in each fraction (**Figure 6**). This correlation followed an exponential decay; moreover, expressing the DP_6 concentration in log scale (semilog plot) results in a sigmoidal scattergram. Data were normalized, and using Prism software a four-parameter logistic curve was fitted to calculate EC_{50} values of 4.40, 5.11, and 6.25% DP_6 relative abundance at 5, 10, and 20 ng/mL Ctx−HRP, respectively, with global $r^2 = 0.859$ and $EC_{50} = 5.10\%$.

DISCUSSION

The decrease in ESI detector response with DP elution is consistent with the findings of Liu et al. (*23*). The spectra of the dextran ladder produced typical Na^+ and K^+ adducts, which is common with ESI in positive polarity mode (*28*). Using negative ion polarity often improves the detection sensitivity because less complex spectra are produced. However, this was not observed in these analyses (data not shown). ZIC-HILIC phase bleed is beneficially low in comparison to other C18 and silica based columns. It was possible to detect 10^4-10^6 ion intensity without observing low-level fragment ions of stationary phase, and therefore sensitivity was greatly improved. Neutral saccharide analysis has routinely been performed using MALDI-MS rather than ESI-MS because underivatized saccharides could not be measured at the same level of sensitivity when compared to proteins and peptides (*29*).

In a recent paper, a fructooligosaccharide (FOS) mixture was efficiently separated into its constituent DP using a HILIC based column (*30*). Generally, few published examples exist of oligosaccharide mixtures having been separated with HILIC before analysis using ESI-MS. One example combined HILIC with ESI-MS to analyze polar compounds from plants. Mass spectrometric fragmentation of the most intense ions was employed to identify unknown compounds, which when elucidated was included into a custom mass library. Using standards, it was possible to quantify constituents of the mixture (*31*). Alpert et al. (*25*) compared HILIC and reverse phase (RP) HPLC to separate sialic acids and hexoses from pea root extract (*Pisum sativum*). Although selectivity of the two methods was complementary, HILIC selectivity was far superior. Changing the ratio of acetonitrile to water in successive experiments decreased the sialic acid residue interaction with the stagnant aqueous mobile phase (*25*). Mechanistic studies of analyte retention on silica-based columns has also been discussed, and it was shown that sensitivity was increased by 3−4 orders of magnitude using HILIC compared to a RP C18 column when used with ESI-MS (*32*). Although there are no reports in the literature where GOS has been separated using HILIC, a recent paper showed separated underivatized oligos with a cyclodextrin-bonded stationary phase column linked to ESI. Both cellooligosaccharides and maltooligosaccharides were also successfully separated at flow rates ranging from 200 to 800 μL/min isocratically with positive ion polarity (*23*).

We propose that reduction in detected DP_6 and DP_5 concentration and corresponding increase in DP_4 and DP_3 concentration appears to affect the inhibitory efficacy using competitive ELISA. The use of IEX to purify multigram quantities of GOS fractions containing just one structure was beyond the scope of the technique because GOS fractions were more complex than anticipated. Size exclusion chromatography would also be impractical, requiring many separations to produce quantities sufficient for bioassay analysis (*33*). Moreover, this is also the first study of GOS using ESI-MS, and despite the less than expected resolution using HILIC, this is the most detailed analysis of the composition of commercial GOS to date.

We correlated profiles of each GOS fraction from HILIC-ESI-MS against the percent inhibition Ctx−HRP bound and found DP_6 structures as the likely inhibitory ligand with a global r^2 value of 0.852. DP_6 abundance was shown to decrease with each fraction from 23% (23.2 μM MW of 990 Da) in GOS fraction 1 to 9% (9.1 μM MW of 990 Da) in GOS fraction 10 and 0% in GOS fraction 16. A simultaneous increase in DP_3 and DP_4 was observed, indicative of a shift from predominantly DP_6 and DP_5 to DP_4 and DP_3 containing fractions. Because the biological activity of each

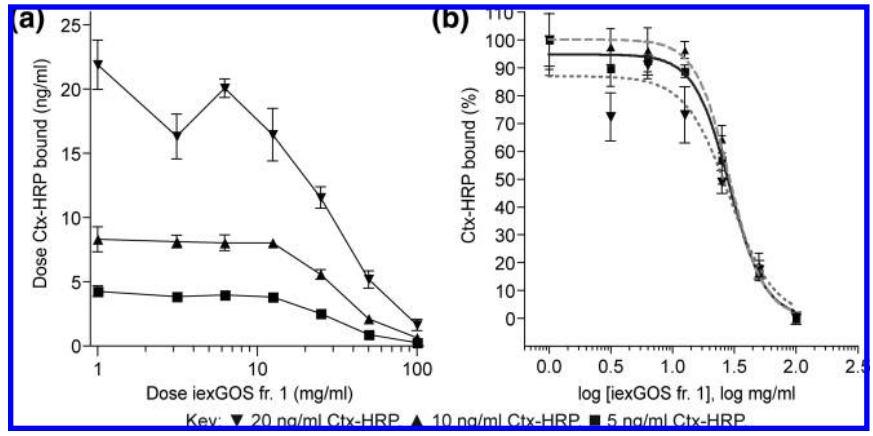

Figure 4. Ctx-HRP inhibitory curves of iexGOS fraction 1 ($n = 6$, $P < 0.001$, $EC_{50} = 29.27$ mg/mL): (▼) 20 ng/mL; (▲) 10 ng/mL; (■) 5 ng/mL. Two-way ANOVA was calculated from log transformed Ctx-HRP bound values using Minitab v14.20 for Windows (Minitab Ltd., Coventry, U.K.). Error bars represent $\pm 1 \times$ SE.

Table 1. EC_{50} Values of Each iexGOS Fraction Calculated from Four-Parameter Logistic Curves Using PRISM v4 Software[a]

iexGOS fraction	EC_{50} value (mg/mL)	global r^2 (20, 10, 5 ng/mL Ctx-HRP)
1	29.27	0.923
2	29.31	0.936
3	27.22	0.957
4	32.03	0.911
5	39.06	0.888
6	39.88	0.801
7	40.39	0.869
8	52.63	0.776
9	56.04	0.605
10	not possible to calculate	not possible to calculate

[a] No statistical difference between 20, 10, and 5 ng/mL Ctx-HRP curves was calculated ($P < 0.001$); therefore, r^2 is representative of all curves (global r^2).

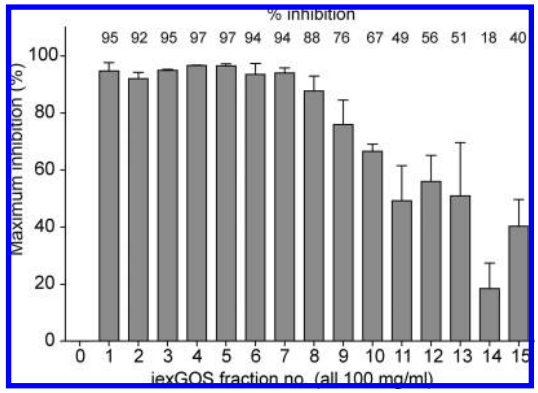

Figure 5. Maximum inhibition of 20 ng/mL Ctx−HRP after incubation with 100 mg/mL iexGOS fractions 1−15 ($n = 6$, $P < 0.001$, $F = 9.60$, $r^2 = 0.7414$). One-way ANOVA was calculated from log transformed Ctx−HRP bound values using Minitab v14.20 software (Minitab Ltd., Coventry, U.K.). Error bars represent $\pm 1 \times$ SE.

fraction was measured using 100 mg/mL, the main difference between each fraction is the relative saccharide abundance. It can be assumed that the EC_{50} value of 5.10% relates to an actual concentration of 5.10 mg/mL (5.15 μM MW of 990 Da). This is a significant improvement compared to data showing galactose reduced the extent of binding to GM1 on liver membranes by only 20% at 200 mM (or 36.04 mg/mL). Furthermore, *N*-acetyllactosamine, glucose, and sialic acid have also been found to be ineffective in inhibiting Ctx binding to GM1 at concentrations of up to 40 mM (*34*).

Whereas the individual contribution of each of the constituent saccharides has low affinity for cholera toxin, GM1-OS multivalency increases this affinity significantly. For example, K_D values of 210, 14.8, and 7.6 mM for sialic acid, galactose, and *N*-acetyllactosamine, respectively, are weak compared to 4.3×10^{-5} mM for GM1-OS (*35*). It is therefore clear that whereas simple saccharides have poor affinities in the millimolar range, weak binding saccharides as part of a larger molecule such as GOS exhibit co-operative affects (*36*).

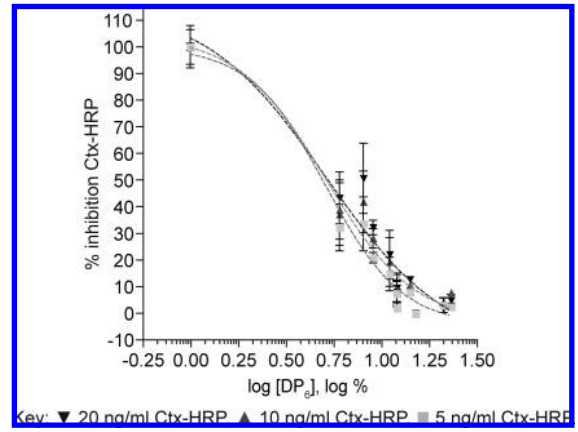

Figure 6. Correlation between the dose of DP_6 and inhibition of Ctx−HRP ($n = 6$, $P = 0.2748$, $r^2 = 0.8516$, $EC_{50} = 5.10\%$ DP_6). Error bars represent $\pm 1 \times$ SE. Four-parameter logistic curve fitted using GraphPad Prism v4.0c (GraphPad Software, San Diego, CA).

High total GOS concentrations are necessary to maintain a DP_6 concentration high enough to fully inhibit Ctx−HRP. This suggests the finding that unfractionated GOS containing glucose, galactose, and lactose does not inhibit Ctx−HRP (not shown). This study demonstrates that fractionation has increased efficacy by concentrating particular GOS isomers the effects of which are otherwise diluted in the commercial GOS formulation. Unfortunately, very little is known about the specific anomeric and linkage configurations in GOS, although the manufacturer, Friesland Foods Domo, suggests the presence of mainly $\beta(1\rightarrow4)$ and $\beta(1\rightarrow6)$ linkages.

There are a number of significant in vitro studies with Ctx and galactosyl residue containing inhibitors. The most relevant concerned fractionated glycolipids from human milk (*37*), the data of which suggested that trace amounts of ganglioside-like structures were one of the protective components of a non-immunoglobulin nature (*37*). The results concurred with an earlier study by Holmgren et al. using both ELISA and rabbit intestinal loops (*38*). The precise identity of this receptor

analogue remains unknown; however, traces of GM1 in human milk have also been detected using a sensitive immunoassay (*39*). A recent study with human milk oligosaccharides (HMO) investigated the inhibition of the diarrheal organisms themselves to Caco-2 epithelial cells (*7*). Specifically, *E. coli* O119, *V. cholerae*, and *S. fyris* binding was inhibited with fractions of HMO produced using gel filtration and characterized into acidic, neutral high molecular weight, and neutral low molecular weight saccharides. In particular, the neutral high molecular weight fraction had the greatest effects on *V. cholerae* and *E. coli* O119 adhesion (*7*). Most recently, 16 mg/mL GOS was shown to reduce adherence of enteropathogenic *E. coli* to HEp-2 and Caco-2 cells (*40*).

Highly sensitive determination of saccharides in complex mixtures is among the most pressing problems of glycobiology generally. Polymeric saccharides can be arranged in many configurations between each monomer, giving rise to a structural diversity that far exceeds that of either DNA or proteins. Consequently, analysis of structures within GOS was equally complex and challenging. There are no reports in the literature of GOS having previously been thoroughly investigated as inhibitors of Ctx. GOS appear to offer a significant promising advantage over simple saccharides, the potency and suitability as a glycomimetic of which could improve further if incorporated into a glycodendritic structure (*41*).

ABBREVIATIONS USED

NH_4OAc, ammonium acetate; BSA, bovine serum albumin; Ctx, *Vibrio cholerae* toxin; Ctx-HRP, *Vibrio cholera* toxin B-subunit conjugated to horseradish peroxidase; DP, degree of polymerization; ESI-MS, electrospray ionization mass spectrometry; gal, galactose; GHP, glucose homopolymer ladder; glu, glucose; GOS, galactooligosaccharides; iexGOS, galactooligosaccharide fraction prepared using cation exchange chromatography; gu, glucose units; HILIC, hydrophilic interaction chromatography; HLT, enterotoxigenic *Escherichia coli* heat labile toxin; HMO, human milk oligosaccharides; IEX, ion exchange chromatography; lac, lactose; oligos, oligosaccharides; RP, reverse phase; TMB, 3,3′,5,5′-tetramethylbenzidine.

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