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Adherence Inhibition of Enteropathogenic *Escherichia coli* by Chitooligosaccharides with Specific Degrees of Acetylation and Polymerization

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ABSTRACT: Some oligosaccharides are known to act as molecular decoys by inhibiting pathogen adherence to epithelial cells. The present study was aimed at analyzing whether chitooligosaccharides (CHOS), that is, oligomers of D-glucosamine and *N*-acetyl-D-glucosamine, have such antiadherence activity. CHOS of varied degree of polymerization (DP) and fraction of acetylation (F_A) were produced. Adherence of enteropathogenic *Escherichia coli* (EPEC) to the surface of a human HEp-2 cell line was determined in the absence or presence of the various CHOS fractions. Adherence was assessed by microscopic counting and image analysis of bacterial clusters and cells. The results showed that all CHOS fractions inhibited adherence of EPEC to HEp-2 cells. Hydrolysates with lower F_A were more effective at reducing adherence. This effect is greater than that obtained with other oligosaccharides, such as galactooligosaccharides, applied at the same concentrations.

KEYWORDS: *oligosaccharides, adherence, chitooligosaccharides, chitosan, chitinase, chitosanase*

INTRODUCTION

Oligosaccharides have long been known to have a variety of biological activities, although the full diversity of their functions is not yet fully understood.[1,2] In particular, they are known to serve as ligands and participate in binding interactions with specific lectins.[2] Recently, it has been suggested that some food grade oligosaccharides can protect host tissue from pathogen adherence.[3] Specifically, galactooligosaccharides (GOS), mannan oligosaccharides, and pectic oligosaccharides have been shown to be effective in inhibiting pathogen binding to the surface of tissue culture cells.[4−7]

For most microbial enteric pathogens, the first step in the infection process is adherence to the epithelial cells that line the intestinal tract. Adherence is generally mediated in these bacteria via expression of lectin-like adhesins that recognize carbohydrate-containing receptor sites on the surfaces of host epithelial cells.[8,9] Accordingly, adherence inhibition may occur in the presence of substances that interfere with the lectin-receptor interaction, for example, by antiadherence oligosaccharides that resemble the glyco-moieties of the host receptor sites. Thus, strategies based on preventing or inhibiting pathogen adherence could be effective at reducing infections and the subsequent onset of disease.[7,10,11]

One group of oligosaccharides that has attracted considerable research and commercial interest due to their biological properties are the chitooligosaccharides (CHOS). CHOS are produced enzymatically or chemically from chitosan; linear heteropolymers of β (1→4) linked *N*-acetyl-D-glucosamine (GlcNAc) and its deacetylated counterpart D-glucosamine (GlcN). Chitosans may have varying compositions, usually indicated by the fraction of acetylated sugar residues (F_A). Soluble chitosans are produced from insoluble chitin by partial or complete *N*-deacetylation, either by homogeneous [12] or by heterogeneous deacetylation.[13] Chitin is an abundant natural product found in nature as a structural component of the cell wall of fungi and yeasts and in the exoskeletons of insects and arthropods (e.g., crabs, lobsters and shrimps). Chitosan has a wide range of applications,[14−16] including its use as an antimicrobial agent.[17−22]

Chitosan can be hydrolyzed by chitinases or chitosanases to give CHOS.[23−26] These hydrolytic enzymes vary with respect to their specific cleavage sites, which are determined by sequences in heteropolymers of GlcNAc and GlcN. Thus, different combinations of chitosans (varying in F_A) and hydrolytic enzymes (varying in sequence specificity) will yield CHOS differing in both length and sequence features.[27] The resulting CHOS are defined by their F_A, their average degree of polymerization (DP_n) and their sequence, that is, the pattern of *N*-acetylated sugar residues (P_A). The DP_n is related to α, a parameter that indicates the degree of scission, where $\alpha = 1/DP_n$. Complete conversion of chitosan to dimers ($DP_n = 2$) would yield an α value of 0.50. Methods exist to separate CHOS by DP (e.g, size exclusion chromatography[26]) and by charge (e.g., cation exchange chromatography[28]). The latter is based on the fraction of deacetylated residues.

CHOS possess a wide range of bioactivities and are used for their antiangiogenesis effects, as well as for wound healing and as vectors in gene therapy.[27,29−32] Chitosan and CHOS are biodegradable and are considered nontoxic;[33] therefore, these

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compounds have a variety of potential applications in food.[34] There is evidence that CHOS (DP < 30, F_A = 0.01−0.12) may be prebiotic, enhancing growth of *Bifidobacterium* and *Lactobacillus* strains in cell cultures.[35,36] This effect is apparently dependent on F_A, since Fernandes et al. [37] reported that CHOS with similar DP but higher F_A (F_A = 0.35) did not stimulate growth of selected strains of bifidobacteria and lactobacilli.

Although pathogen adherence by CHOS has received relatively little attention, one previous study showed that a nondefined CHOS mixture of F_A = 0.03 and $DP_n \approx 4$ inhibited adherence of three different strains of enteropathogenic *Escherichia coli* (EPEC) on HT-29 cells.[38] In addition, Liu et al. showed that weaned pigs that were fed CHOS exhibited reduced incidence of diarrhea after being challenged with *E. coli* K88.[39] It is now possible, however, to produce more defined CHOS fractions and to assess these fractions for antiadherence activity. Thus, the main objective of the present study was to test CHOS with different F_A and DP for their ability to inhibit adherence of EPEC, a widely recognized enteric pathogen, on tissue culture cells.

MATERIALS AND METHODS

Preparation of CHOS. Three chitosans with different F_A were enzymatically hydrolyzed. A chitosan with F_A = 0.15 (KitoNor from Norwegian Chitosan, Gardermoen, Norway) and a chitosan with F_A = 0.3 (Heppe Medical Chitosan GmbH, Halle, Germany) were hydrolyzed with purified recombinant chitosanase ScCsn46A from *Streptomyces coelicolor* A3(2).[23] A F_A0.65 chitosan was prepared by homogeneous deacetylation of chitin from shrimp shells [12] (Chitinor, Senjahopen, Norway). This F_A0.65 chitosan was hydrolyzed with purified recombinant ChiB from *Serratia marcescens*.[26,40] The F_A of the chitosans before enzymatic hydrolysis, and the degree of scission (α) after degradation were determined by ^{1}H NMR using a Varian Gemini instrument at 300 MHz.[26,41]

The F_A0.65 chitosan was soluble in water, whereas the F_A0.15 and F_A0.3 chitosans required 0.5% acid to dissolve. All three chitosans were dissolved/suspended in buffer (40 mM NaAc, 100 mM NaCl, pH 5.5) to a concentration of 10 mg/mL. Then, 0.5% (v/v) 12 M HCl was added to the F_A0.15 and F_A0.3 chitosan samples, and after the chitosan was dissolved, the pH was adjusted to 5.5 with 6 M NaOH. Enzymes were added to prewarmed chitosan solutions to a final concentration of 0.5 μg/mg chitosan and the reactions, with a final chitosan concentration of approximately 9.8 mg/mL, were incubated at 37 °C with shaking (225 rpm). Reactions were stopped by decreasing the pH to 2.5 with HCl. The CHOS samples were filtered through Filtropur S 0.2 μm sterile filters (Sarstedt, Germany), lyophilized and resuspended in the size exclusion chromatography (SEC) mobile phase to a concentration of 20 mg/mL prior to separation on SEC.

Separation of CHOS. The CHOS were separated by size exclusion chromatography (SEC) on three XK 26 columns packed with Superdex 30 prep grade (GE Healthcare) coupled in series with an overall dimension of 2.6 cm × 180 cm. The mobile phase (150 mM NH_4Ac, pH 4.6) was run at a constant flow of 0.8 mL/min.[26] The column eluent was monitored using an RI detector (Gilson model 133). In each run 100 mg of chitosan hydrolysate was applied (i.e., 5 mL) and 3.2 mL fractions were collected. Identification of oligomers in the fractions was performed with MALDI-TOF-MS. The fractions were dialyzed with Float-A-Lyzers (MWCO 100−500 Da, SpectrumLabs) to remove salts, sterile filtrated and lyophilized. Prior to use, the CHOS were dissolved in sterile distilled water.

To limit the number of assays, initial experiments were done with chitosan hydrolysates containing mixtures of CHOS. In this case, dried material was resuspended in sterile water to a final volume of 1 mL (final concentration varied according to the amount available of each sample). For other experiments, samples were diluted to a final concentration of 16 mg/mL.

Strains and Organisms. EPEC strain E2348/69 (O127:H6) was obtained from M. Donnenberg (University of Maryland School of Medicine, Baltimore) and was used as a model organism for the antiadherence experiments. Before each experiment, cells from frozen stocks were plated on tryptic soy agar (TSA; Difco, Sparks, USA) and grown overnight at 37 °C, as described previously.[7] A single colony was then inoculated into 10 mL of tryptic soy broth (TSB; Difco) and incubated overnight at 37 °C without shaking. Overnight cultures were used to inoculate (1% v/v) minimal essential medium (MEM; Hyclone, Logan, UT) supplemented with 10% (v/v) fetal bovine serum (FBS; Hyclone, Logan, UT). MEM was pre-equilibrated overnight at tissue culture conditions (5% CO_2, 95% relative humidity, 37 °C). The cells were then incubated for 80 min at 37 °C, aerobically, prior to the start of the experiment.

Tissue Culture Cells. HEp-2 (CCL-23) cells were obtained from the American Type Culture Collection (Manassas, VA). This cell line was used to assess bacterial adherence to epithelial cells; they are a widely used cell line for studies involving bacterial intestinal adherence.[42−44] HEp-2 cells were grown as described previously.[7] Briefly, cells were grown in 75 cm^2 tissue culture flasks containing 25 mL of MEM (pH 7.4) supplemented with 10% FBS in a CO_2 incubator at tissue culture conditions. Confluent HEp-2 cells were harvested by removing MEM and washing the cells once with PBS. Subsequently, 0.5 mL of a 0.25% Trypsin-EDTA solution was added followed by a 10 min incubation at tissue culture conditions. After incubation, 0.5 mL of FBS was added to inactivate the trypsin. Cells were then seeded onto 12 mm diameter glass coverslips in 24-well tissue culture plates at approximately 3.6×10^5 cells per well, and 500 μL of MEM supplemented with 10% FBS was added to each well. Plates were incubated under tissue culture conditions for about 20 h prior to the start of each experiment. Cells were checked before the experiment under an inverted microscope to make sure they had reached about 70% confluency.

Antiadherence Assays. CHOS were dissolved in sterile water and mixed with bacterial cultures (approximately 10^8 cells/mL in MEM supplemented with 10% FBS) to final concentrations of 16 mg/mL, prior to addition to the tissue culture cells. A total of 14 fractions were analyzed - three CHOS mixtures resulting from enzymatic hydrolysis of three different chitosans (F_A0.15, F_A0.3, and F_A0.65) and 11 fractions derived from hydrolyzed F_A0.15 chitosan by size exclusion. In addition, a mixture of *N*-acetyl-D-glucosamine (Sigma) and D-glucosamine (Sigma) (15:85) was used as a control. It was not possible to test the nonhydrolyzed chitosans, due to the viscous nature of the chitosan solutions. Sterile water was also used as a control. The standard CHOS concentration of 16 mg/mL was used because previous experiments with GOS had shown this concentration to be effective in inhibiting adherence of EPEC.[7] However, for some of the fractions, the amount of material was limited, and the concentration used was significantly lower. In addition, dose responses of the fractions F_A0.15 and F_A0.3 were performed to determine the effect of concentration on adherence inhibition.

After addition of the bacteria/CHOS mixtures to tissue culture cells, the plates were incubated for 30 min at tissue culture conditions (as described above). The wells were then washed five times with PBS to remove nonadhered bacteria. Cells were then fixed with 100% methanol and stained with 10% Geimsa. Glass coverslips with stained cells were mounted on microscope slides and analyzed by phase contrast microscopy (100×) with an attached camera. A predetermined horizontal and vertical pattern was established to obtain fifteen images of each coverslip. Bacterial clusters (defined as bundles of 4 or more bacteria) and HEp-2 cells were counted using ImageJ software to obtain a ratio of bacterial clusters/100 HEp-2 cells. Single concentration experiments were replicated once (n = 2) and dose responses were replicated five times (n = 5). The % inhibition was calculated as the number of adhered clusters in the control minus the number of adhered clusters in the treatment, all divided by the number of adhered clusters in the control. Thus, 0% inhibition would refer to the control containing only water. Because EPEC cluster formation occurs via bundles of 4 or more bacteria, microscopy is the preferred

method for quantifying adherence of this organism. It is also used to assess the manner of cell attachment of other bacteria.[7,45]

RESULTS

Production, Separation and Characterization of CHOS. In the initial experiments, chitosans with F_A0.15 and F_A0.3 were enzymatically hydrolyzed with chitosanase SnCsn46A from *Streptomyces coelicolor* A3(2) to α = 0.22 and 0.25, respectively (corresponding to DP_n values of 4.5 and 4.0, respectively). The F_A0.65 chitosan was enzymatically hydrolyzed by ChiB from *Serratia marcescens* to α = 0.19 (DP_n 5.3). The α values were determined by ^{1}H NMR as described previously [31] and SEC chromatograms of the samples (not shown) confirmed that, as expected, the majority of the CHOS was in the DP 2−20 range.

Since the F_A0.15 sample was the most inhibitory in the initial experiments (see below), a new hydrolysis reaction was set up (α = 0.16, DP_n 6.3) and the CHOS were separated into single fractions (DP3−DP12) and one fraction with DP > 12 and a DP_n of 25 (Figure 1A). The MALDI-TOF-MS spectra of the individual DP3−DP12 fractions demonstrate that the various fractions were generally homogeneous with respect to DP (Figure 1B and 1C). The mass spectra also give an impression of the F_A distributions within the samples. For example, the DP4 fraction contains primarily D_4 and D_3A_1, the DP6 fraction contains D_6, D_5A_1 and D_4A_2, and the DP12 fraction contains D_{12}, $D_{11}A_1$, $D_{10}A_2$, D_9A_3 and D_8A_4.

Inhibition of EPEC Adherence by CHOS Mixtures with F_A0.15, F_A0.3 and F_A0.65. The nonseparated hydrolysates of chitosans with F_A = 0.15, 0.3 and 0.65 were tested for their ability to inhibit EPEC adherence at a concentration of 16 mg/mL, a concentration used in previous studies for other prebiotic oligosaccharides.[7,45] In addition, the adherence inhibition activity of fractions F_A0.15 and F_A0.3 was also assessed over a range of concentrations (0, 0.5, 1, 5, 10, and 16 mg/mL). Microscopic analysis revealed that EPEC adherence was reduced by the F_A0.15 fraction (Figure 2), and by image analysis, all three hydrolysates significantly inhibited adherence (Figure 3C). For both fractions F_A0.15 and F_A0.3, a dose-dependent trend was observed (Figure 3A and 3B). Comparison of CHOS based on F_A revealed that the F_A0.15 fraction gave the highest inhibition (92%) as compared to the F_A0.65 sample (75%) and the F_A0.3 sample (84%). It was not possible, however, to assess the activity of nonhydrolyzed chitosans because the high viscosity of the chitosan solutions interfered with adherence experiments. Bacterial motility is reduced in highly viscous solutions, resulting in obstruction of bacteria from coming in contact with the tissue culture cells.

Inhibition of EPEC Adherence by Purified CHOS Fractions. Single fractions of CHOS purified from hydrolyzed F_A0.15 chitosan as described above (Figure 1) were then tested in the same EPEC adherence assay. The fractions tested were single fractions of DP3 to DP12, and a mixture with DP > 12 and DP_n = 25. All CHOS fractions significantly inhibited adherence compared to the control, reaching inhibition levels of close to 100% (Figure 4). Notably, adherence was not inhibited by addition of a 15:85 mixture of the monomers, GlcNAc and GlcN (DP1 in Figure 4), indicating that the oligomeric nature of the sugars is essential for the inhibitory effect.

Growth of EPEC in the Presence of CHOS, GlcNAc and GlcN. EPEC was grown in TSB medium containing monomers o·f GlcNAc, GlcN, and a 15:85 mixture of these sugars, all at a

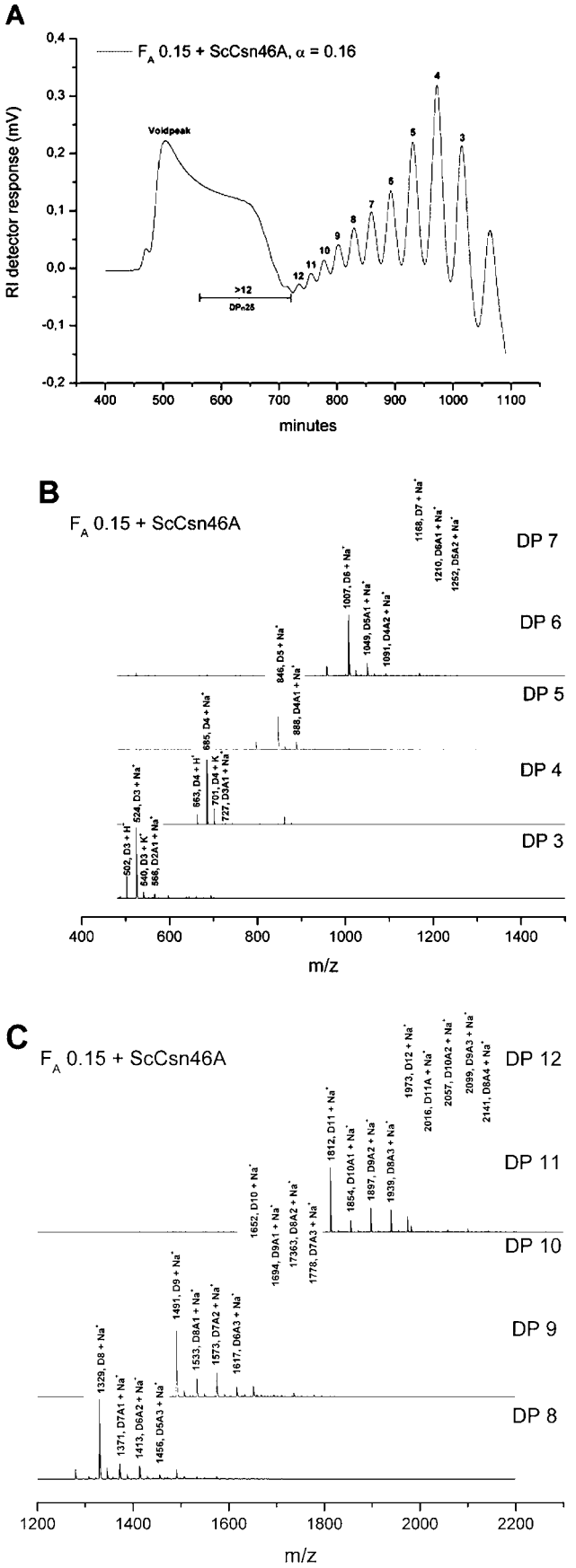

Figure 1. SEC and MALDI-TOF analysis of hydrolyzed F_A0.15 chitosan. (A) Size exclusion chromatogram (SEC) of CHOS obtained by enzymatic hydrolysis of the F_A0.15 chitosan with ScCsn46A from

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Figure 1. continued

Streptomyces coelicolor A3(2). Peaks are labeled by the DP of the oligomers they contain; the region labeled ">12" and "DP_n25" was collected and tested as one (mixed) fraction. MALDI-TOF-MS analysis was performed on the different SEC fractions. The (B) DP3−7 fractions and (C) DP8−12 fractions are shown. Major signals are labeled by mass, sugar composition (A, GlcNAc; D, GlcN) and adduct type (H^+, Na^+ or K^+).

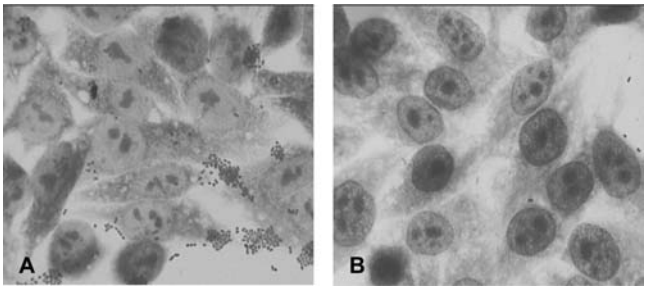

Figure 2. Micrographs (100× magnification) of EPEC adherence to HEp-2 cells in the (A) absence and (B) presence of CHOS with F_A = 0.15 at a concentration of 16 mg/mL.

concentration of 16 mg/mL (i.e., the same concentration used in the CHOS antiadherence assays). Growth of EPEC was not impaired by the presence of any of these monomers or the mixture (Figure 5). A similar experiment with one of the CHOS fractions also showed that growth of EPEC was unaffected by CHOS in the media. In addition, the bacterial inoculum was enumerated before and after incubation to ensure there was no reduction due to a potential bactericidal effect of the CHOS (data not shown).

DISCUSSION

The use of molecular decoys as antiadherence agents was proposed more than a decade ago.[8,46−48] In recent years, several food grade prebiotic oligosaccharides and plant extracts have been tested for their ability to inhibit pathogen adherence to the surface of intestinal epithelial cells. In a previous study, we showed that GOS inhibited EPEC adherence by up to 65% under conditions similar to those used in the present study.[7] Interestingly, the results indicate that CHOS, especially those with low F_A, are more effective inhibitors of EPEC adherence than GOS, given that inhibition reached almost 100% for some of the fractions tested.

Our results also showed that CHOS with different F_A, but similar DP_n (4.0−5.3) had comparable adherence inhibition activities, with low F_A being the most effective (Figure 3C). Thus, the glucosamine content, which affects charge density due to the titratable amino group of this sugar, appears to affect the activity of CHOS against EPEC adherence. Furthermore, a dose-dependent antiadherence effect was observed, as greater adherence inhibition occurred at the higher concentrations, until a plateau was reached. In addition, the results showed that hydrolysates with different F_A require different concentrations to reach the same level of inhibition (Figure 3A and 3B). Adherence inhibition, however, did not appear to be related to DP, as CHOS fractions purified from a hydrolyzed F_A0.15 chitosan, but with DP's ranging from 3 to greater than 12, all inhibited adherence by up to 99%. Nevertheless, it should be

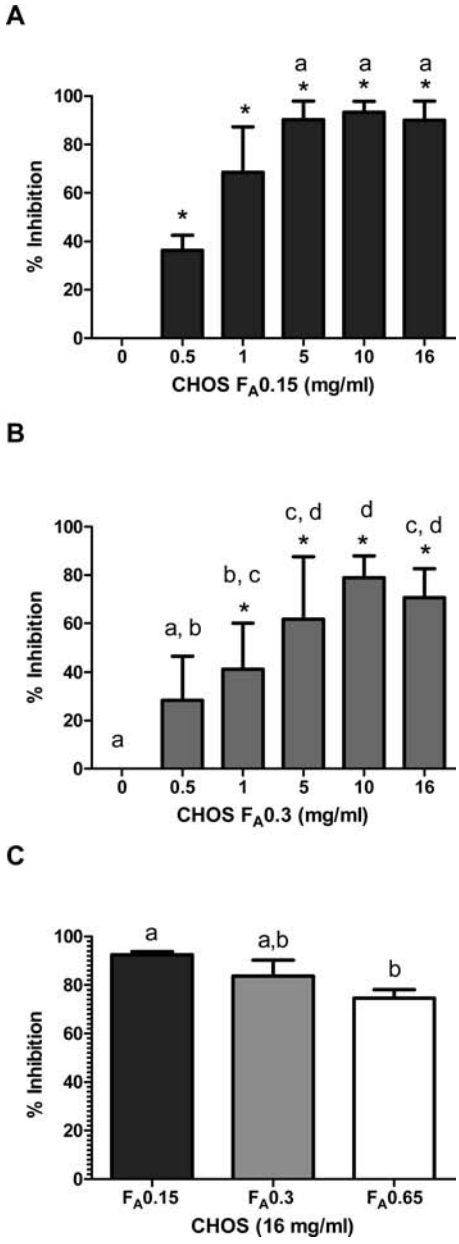

Figure 3. Inhibition of EPEC adherence to HEp-2 cells by CHOS mixtures with different F_A. The % Inhibition was calculated as described in the text. Statistical analysis was performed by Analysis of Variance (ANOVA) to determine statistical differences from the control, and Tukey's test was used to determine significant differences among the treatments. Values sharing the same letter are not significantly different from each other ($p < 0.05$) For (A) F_A0.15 and (B) F_A0.3 dose experiments, n = 5; (C) for comparison between F_A0.15, F_A0.3, and F_A0.65, n = 2.

noted that CHOS with different DPs will have different molecular weights, hence contributing a different number of target molecules despite being used at the same concentration.

The antiadherence property of oligosaccharides has been attributed to the similarity between the oligosaccharide structure and cell surface receptor to which bacteria attach prior to colonization. Via a phenomenon known as phase variation,[49] bacteria can modulate adhesin expression, depending, in part, on the available receptors expressed by the host cells. This may account for why some oligosaccharides are effective in inhibiting adherence of particular pathogens

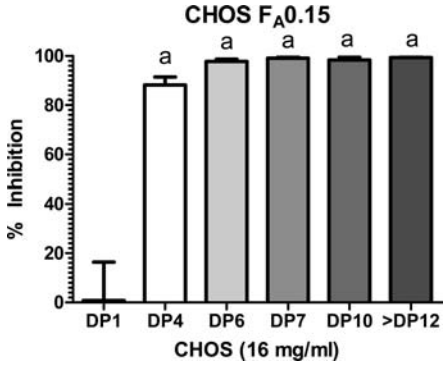

Figure 4. Inhibition of EPEC adherence to HEp-2 cells by purified CHOS fractions with different DP. Statistical analysis was performed by Analysis of Variance (ANOVA) to determine statistical differences from the control, and Tukey's test was used to determine significant differences among the treatments (n = 2). Groups sharing the same letter are not significantly different from each other (p < 0.05).

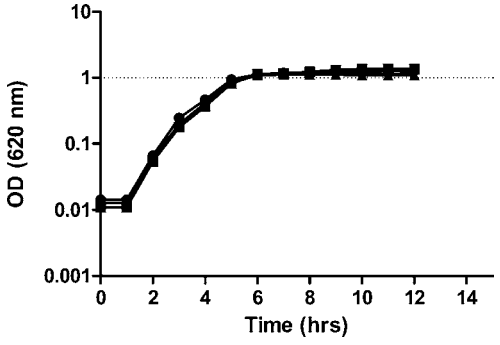

Figure 5. Growth of EPEC in the presence and absence of GlcN and GlcNAc. Growth of EPEC in TSB at 37 °C was measured in the absence (●) or in the presence of added sugars. Sugars tested were GlcN (■), GlcNAc (▲) and a 85:15 mix of GlcN:GlcNAc (▼), all at a total sugar concentration of 16 mg/mL.

whereas others are not affected. Moreover, the molecular interaction between oligosaccharides and bacterial adhesins varies among different pathogens, and in some cases among different strains.[7,45]

Recently, it was suggested that pathogen adherence tropism is dependent on three key elements: expression of adhesins; adhesion specificity; and the presence of cognate receptors on the surface of specific tissue culture cells.[50] Although the precise mechanism for how CHOS prevent adherence of EPEC to epithelial cells will require further investigation, we suggest that CHOS interferes with adhesion attachment to the cognate ligands. In particular, one of the monomers of CHOS is GlcNAc, which is a common constituent of receptor ligands for many bacterial lectins.[51−53] However, the occurrence of nonacetylated glucosamines as a target ligand on the surface of epithelial cells has not been reported. In addition, the present data clearly shows that inhibition of adherence requires an oligomeric carbohydrate (Figure 4), as free monomeric sugars had no effect on adherence.

The ability of chitosan polymers to inhibit growth of *E. coli* has been reported previously,[19,54,55] although this effect was observed only for chitosans of higher DP, that is, higher than the DP of the CHOS used in the present study. Other studies have shown that shorter CHOS, at DP < 20 do not kill *E. coli*.[20,55] Indeed, growth of EPEC was not impaired by the CHOS used in this study, indicating that reduced adherence of EPEC was not due to growth inhibition or cell killing. Thus, it seems that the antiadherence effect of CHOS is independent of the other biological effects of CHOS and chitosan.

In summary, our results show that different fractions of CHOS inhibit adherence of EPEC to the surface of tissue culture cells. Further research is needed to identify the specific CHOS species responsible for the observed inhibition and to assess these effects *in vivo*, that is, on pathogen adherence in the animal gastrointestinal tract. Finally, other potential biological activities of CHOS, including their possible impact on the intestinal commensal microbiota, should also be considered. Certainly, nonpathogenic strains of *E. coli* may also express adhesins and bind to CHOS or other molecular decoys. However, provided that the concentration of the antiadherence agents is sufficient, inhibition of targeted pathogens would still be expected to occur.

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Notes

The authors declare no competing financial interest.

ABBREVIATIONS

CHOS, Chitooligosaccharides; DP, degree of polymerization; EPEC, enteropathogenic *Escherichia coli*; F_A, fraction of acetylation; TSB, tryptic soy broth; TSA, tryptic soy agar; MEM, minimal essential medium

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