Adherence inhibition of enteropathogenic *Escherichia coli* by chitooligosaccharides with specific degrees of acetylation and polymerization

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Abstract

Some prebiotic oligosaccharides are known to act as molecular decoys by inhibiting pathogen adherence to epithelial cells. The present study was aim**e**d at analyzing whether chitooligosaccharides (CHOS), i.e. oligomers of D-glucosamine and *N*-acetyl-D-glucosamine, have such anti-adherence 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 studied by adding bacteria to HEp-2 cells grown on glass coverslips, in the absence or presence of the various CHOS fractions. Adherence was assessed by microscopic counting of bacteria and cells. The results show that CHOS inhibit adherence of EPEC to HEp-2 cells by more than 90%. This effect is greater than that obtained with other oligosaccharides, such as galactooligosaccharides, applied at the same concentrations.

Abbreviations

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

Keywords

Prebiotics, adherence, chitooligosaccharides, chitosan, chitinase, chitosanase

Highlights

1. CHOS with defined degrees of polymerization and acetylation were produced.
2. CHOS inhibits adhesion of *E. coli* to HEp-2 cells.
3. Anti-adherence activity increases with decreasing F_A of the CHOS.
4. DP does not seem to have an effect on antiadherence.
5. CHOS could be added to foods as a prophylactic treatment to prevent EPEC infections.

1. Introduction

Prebiotics were originally defined more than 15 years ago as "non-digestible food ingredient(s) that beneficially affect the host by selectively stimulating the growth and/or activity of one or a limited number of bacteria in the colon, and thus improve host health" (Gibson & Roberfroid, 1995). Despite slight modifications of this definition (Roberfroid, 2007), establishing a substance as a prebiotic still depends on the ability of that substance to produce positive changes in the gut microbiota. Recently, it has been suggested that some prebiotic oligosaccharides exert their effects by directly protecting the host from pathogens. Specifically, galactooligosaccharides, mannan-oligosaccharides, and pectic oligosaccharides have been shown to interfere with the ability of pathogenic bacteria to attach to the surface of host tissues (Ganan et al., 2010; Ghosh & Mehla, 2012; Kunz et al., 2000; Shoaf et al., 2006). 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 (Ofek & Beachey, 1978; Ofek, Hasty, & Doyle, 2003). Accordingly, adherence inhibition may occur in the presence of substances that interfere with the lectin-receptor interaction, for example, by prebiotic 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 (Bavington & Page, 2005; Klemm, Vejborg, & Hancock, 2010; Shoaf et al., 2006).

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 acetylation (F_A). Soluble chitosans are produced from insoluble chitin by partial or complete *N*-deacetylation, either by homogenous (Sannan, Kurita, & Iwakura, 1975) or by heterogeneous deacetylation (Rigby, 1934). 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 (Alishahi & Aïder, 2011;

Jayakumar et al., 2010; R. Muzzarelli, 1996), including its use as an antimicrobial agent (Devlieghere, Vermeulen, & Debevere, 2004; Helander et al., 2001; Liu et al., 2004; Mellegård et al., 2011; Rabea et al., 2003; Tsai et al., 2002).

Chitosan can be hydrolyzed by chitinases or chitosanases to give CHOS (Heggset et al., 2010, 2012; Heggset et al., 2009; Sørbotten et al., 2005). 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 (Aam et al., 2010). The resulting CHOS are defined by their F_A, their average degree of polymerization (DP_n) and their sequence, i.e. 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 (Sørbotten et al., 2005)) and by charge (e.g., cation exchange chromatography (Haebel, Bahrke, & Peter, 2007)). The latter is based on the fraction of deacetylated residues.

CHOS possess a wide range of bioactivities including antifungal, antibacterial, and anti-angiogenesis effects, as well as beneficial effects on wound healing and as vectors in gene therapy (Aam et al., 2010; Benhabiles et al., 2012; Muzzarelli, 2005; Wu et al., 2012; Xia et al., 2011) . Chitosan and CHOS are biodegradable and are considered non-toxic (Kean & Thanou, 2010); therefore, these compounds have a variety of potential applications in food (No et al., 2007). There is evidence that CHOS ($DP < 30$, F_A0.01 - 0.12) may be prebiotic, enhancing growth of *Bifidobacterium a*nd *Lactobacillus* strains in cell cultures (Lee et al., 2002; Simůnek et al., 2010). This effect is apparently dependent on F_A, since Fernandes *et al.* (Fernandes et al., 2012) reported that CHOS with similar DP but higher 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 non-defined CHOS mixture of F_A0.03 and $DP_n \sim 4$ inhibited adherence of three different strains of enteropathogenic *Escherichia coli* (EPEC) on HT-29 cells (Rhoades et al., 2006). It is now possible, however, to produce more defined CHOS fractions

and to assess these fractions for anti-adherence 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.

2. Materials and Methods

2.1 Preparation of CHOS

Three chitosans with different F_A were enzymatically hydrolyzed. A chitosan with F_A0.15 (KitoNor from Norwegian Chitosan, Gardermoen, Norway) and a chitosan with F_A0.3 (Heppe Medical Chitosan GmbH, Halle, Germany) were hydrolyzed with purified recombinant chitosanase ScCsn46A from *Streptomyces coelicolor A3(2)* (Heggset et al., 2010). A F_A0.6 chitosan was prepared by homogenous deacetylation of chitin from shrimp shells (Chitinor, Senjahopen, Norway) (Sannan et al., 1975). This F_A0.6 chitosan was hydrolysed with purified recombinant ChiB from *Serratia marcescens* (Bruberg et al., 1995; Sørbotten et al., 2005). 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 (Sørbotten et al., 2005; Vårum et al., 1991).

The F_A0.6 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 chitosans samples, and after the chitosan was dissolved, the pH was adjusted to 5.5 with 6 M NaOH. Enzymes were added to pre-warmed 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.

2.2 Separation of CHOS

The CHOS were separated by size exclusion chromatography (SEC) on three XK 26 columns packed with Superdex^{TM} 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 (Sørbotten et al., 2005). 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.

2.3 Strains and Organisms

EPEC strain E2348/69 (O127:H6) was used as a model organism for the anti-adherence experiments. Before each experiment, cells from frozen stocks were plated on tryptic soy agar (TSA; Difco) and grown overnight at 37°C. 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) supplemented with 10% (v/v) fetal bovine serum (FBS). 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.

2.4 Tissue Culture Cells

HEp-2 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 minute 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 hours 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.

2.5 Anti-adherence Assays

CHOS dissolved in sterile water were mixed with bacterial cultures (approx. 10^8 cells per ml in MEM supplemented with 10% FBS) to final concentrations of 16 mg/ml CHOS, 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_A0.6) 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. We were not able to test the non-hydrolyzed 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 galactooligosaccharides had shown this concentration to be effective in inhibiting adherence of EPEC (Shoaf et al., 2006). However, for some of the fractions the amount of material was limited, and the concentration used was significantly lower.

After addition of the bacteria-CHOS mixtures to tissue culture cells, the plates were incubated for 30 minutes at tissue culture conditions (as described above). The wells were then washed five times with phosphate buffered saline (PBS) to remove non-adhered bacteria. Cells were then fixed with 100% methanol and stained with 10% Geimsa. Glass coverslips with stained cells were mounted on microscope slides to be analyzed under the microscope. Fifteen pictures were obtained for each cover slip and images were analyzed with ImageJ software. Bacterial clusters and HEp-2 cells were counted per image to obtain a ratio of bacterial clusters/HEp-2 cell. Experiments were performed in duplicate and replicated once (n=4).

3. Results

3.1 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_A0.6 chitosan was enzymatically hydrolysed by ChiB from *Serratia marcescens* to α = 0.19 (DP_n 5.3). The α values were determined by NMR as described previously (Wu et al., 2012) and SEC chromatograms of the samples (not shown) confirmed that, as expected, the majority of the CHOS was in the DP 2-20range.

Since the F_A0.15 sample was the most inhibitory in the initial experiments (Fig. 3; discussed below), a new hydrolysis reaction was set up (α = 0.16, DP_n 6.3) and the CHOS were separated into single fractions (DP 3 – DP12) and one fraction with DP > 12 and a DP_n of 25 (Fig. 1A). Fig. 1B and 1C show the MALDI-TOF-MS spectra of the individual DP3-DP12 fractions and demonstrate that the various fractions are quite homogeneous in terms of DP. 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} and $D_{11}A_1$, $D_{10}A_2$, D_9A_3 and D_8A_4.

3.2 Inhibition of EPEC adherence by CHOS mixtures with F_A0.15, F_A0.3 and F_A0.65

The non-separated hydrolysates of chitosans with F_A0.15, F_A0.3, and F_A0.65, 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 (Quintero et al., 2011; Shoaf et al., 2006). The three samples significantly inhibited adherence (see microscopic image in Fig. 2), by as much as 92%, 84% and 75%, respectively. The F_A0.15 CHOS sample gave the highest inhibition; its effect was significantly different from the effect of the F_A0.65 sample ($p < 0.05$), but not from the effect of F_A0.3 sample (Fig. 3). Non-hydrolyzed chitosans could not be tested 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.

3.3 Inhibition of EPEC adherence by purified CHOS fractions

Single fractions of CHOS purified from hydrolyzed F_A0.15 chitosan as described above (Fig. 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. Typical results are shown in Figure 4. All CHOS fractions significantly inhibited adherence compared to the control, reaching inhibition levels of close to 100 % for CHOS with DP6 and higher. Notably, adherence was not inhibited by addition of a 15:85 mixture of the monomers, GlcNAc and GlcN, showing that the oligomeric nature of the sugars is essential for the inhibitory effect.

3.4 Growth of EPEC in the presence of CHOS, GlcNAc and GlcN

EPEC was grown in TSB medium containing monomers of GlcNAc, GlcN, and a 15:85 mixture of these sugars, all at a concentration of 16 mg/ml (i.e. the same concentration used in the CHOS anti-adherence assays). Growth of EPEC was not impaired by the presence of any of these monomers or the mixture (Fig. 5). A similar experiment with one of the CHOS fractions also showed that growth of EPEC was unaffected by CHOS (data not shown).

4. Discussion

The use of molecular decoys as anti-adherence agents was proposed more than a decade ago (Andersson et al., 1986; Cravioto et al., 1991; Ebrahim, 1997; Ofek & Beachey, 1978). 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 galactooligosaccharides inhibited EPEC adherence by up to 65% under conditions similar to those used in the present study (Shoaf et al., 2006). Interestingly, the present 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 chitosans 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 (Fig. 3). 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. However, adherence

inhibition was not related to DP, as CHOS fractions purified from a hydrolyzed F_A0.15 chitosan, but with varying DP's, ranging from 3 to greater than 12, all inhibited adherence by up to 99%.

The anti-adherence 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 (Thanassi, 2011), 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 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 (Quintero et al., 2011; Shoaf et al., 2006).

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 (Korea, Ghigo, & Beloin, 2011). 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 (Buts et al., 2004; Sharon, 1987; Sharon, 2006). However, the occurrence of non-acetylated 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 (Fig. 3), as free monomeric sugars had no effect on adherence.

The ability of chitosan polymers to inhibit growth of *E.coli* has been reported previously, (Eaton et al., 2008; Li et al., 2010; Liu et al., 2004), although this effect was observed only for chitosans of higher DP, i.e. 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* (Li et al., 2010; Mellegård et al., 2011). 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 anti-adherence 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*, i.e., on pathogen adherence in the animal gastrointestinal tract. Finally, other potential biological activities of CHOS, including their possible impact on the intestinal microbiota, should also be considered.

Acknowledgements

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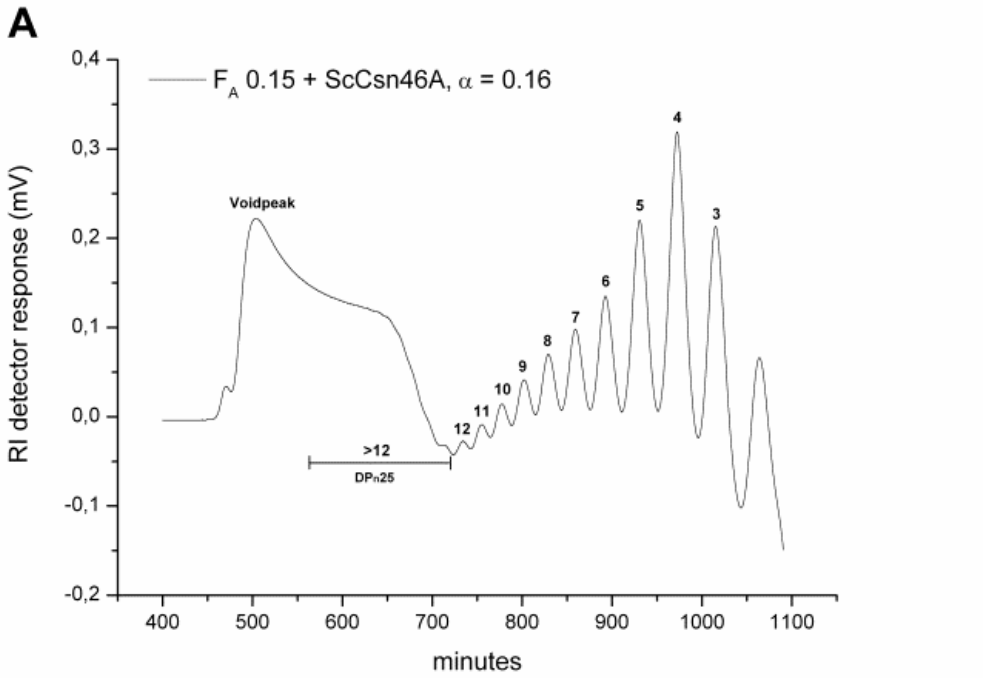
Figures


A
F_A 0.15 + ScCsn46A, α = 0.16
RI detector response (mV)
0,4
0,3
0,2
0,1
0,0
-0,1
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Voidpeak
4
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400
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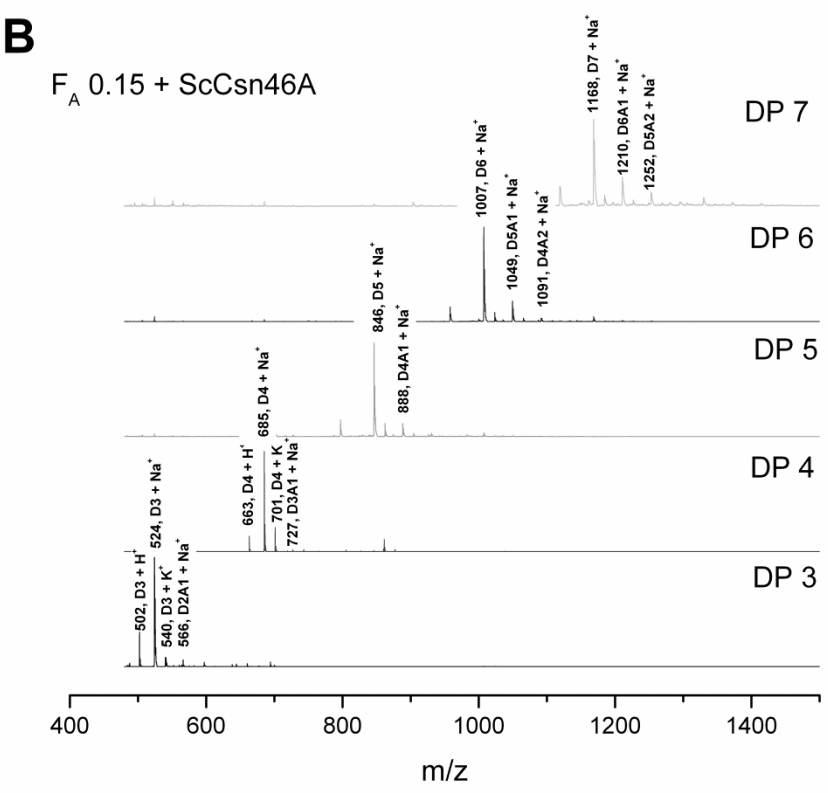
B
F_A 0.15 + ScCsn46A
DP 7
DP 6
DP 5
DP 4
DP 3
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1210, D6A1 + Na+
1252, D5A2 + Na+
1007, D6 + Na+
1049, D5A1 + Na+
1091, D4A2 + Na+
846, D5 + Na+
888, D4A1 + Na+
685, D4 + Na+
663, D4 + H+
701, D4 + K+
727, D3A1 + Na+
502, D3 + H+
524, D3 + Na+
540, D3 + K+
566, D2A1 + Na+
400
600
800
1000
1200
1400
m/z

341

342

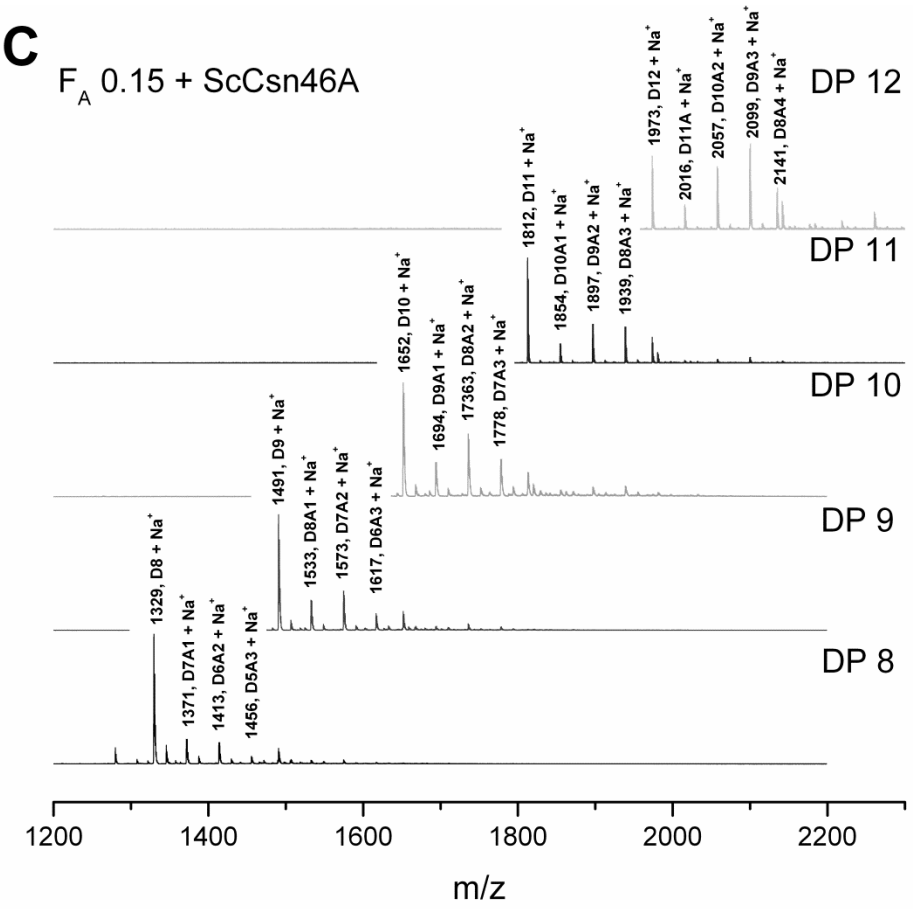

Fig. 1. SEC and MALDI-TOF analysis of hydrolyzed F_A0.15 chitosan. Fig. 1A shows the size exclusion chromatogram (SEC) of CHOS obtained upon enzymatic hydrolysis of the F_A0.15 chitosan with ScCsn46A from *Streptomyces coelicolor A3(2)*. Peaks are labeled by the DP of the oligomers they contain; the 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. Fig. 1B shows the DP 3-7 fractions and Fig. 1C the DP 8-12 fractions. Major signals are labeled by mass, sugar composition (A, GlcNAc; D, GlcN) and adduct type (H^+, Na^+ or K^+).

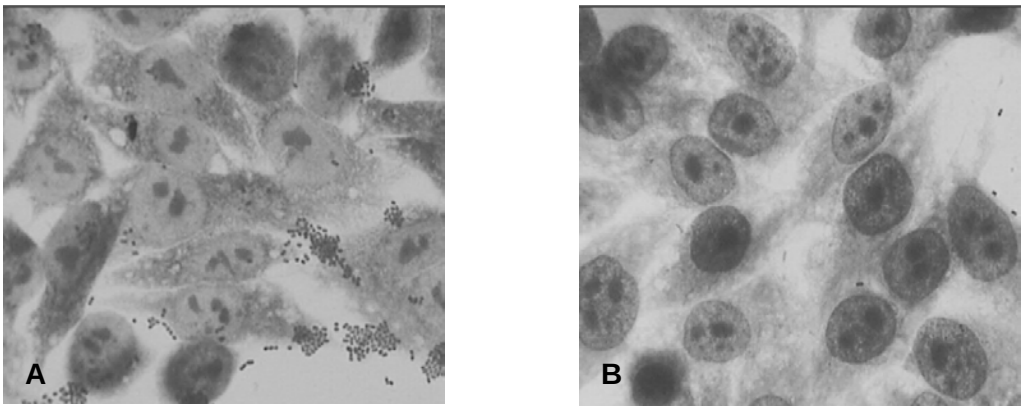

Figure 2. Adherence of EPEC to HEp-2 cells in the presence (B) and absence (A) of CHOS with F_A0.15 at a concentration of 16 mg/ml.

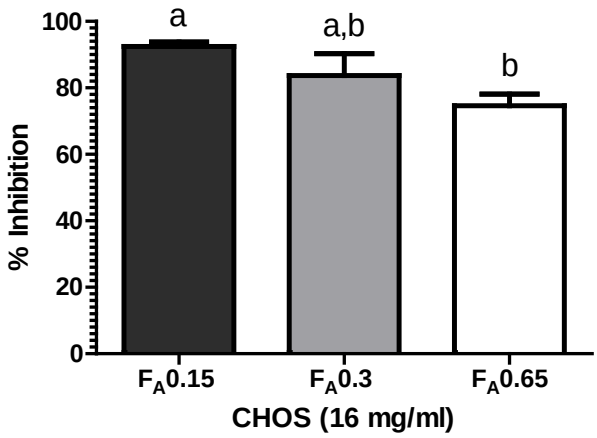

Fig. 3.Inhibition of EPEC adherence to HEp-2 cells by CHOS mixtures with different F_A. Statistical analysis was performed by Analysis of Variance (ANOVA) to determine statistical differences from the control. Tukey's test was used to determine significant differences among the treatments.(n = 4). Values sharing the same letter are not significantly different from each other ($p < 0.05$).

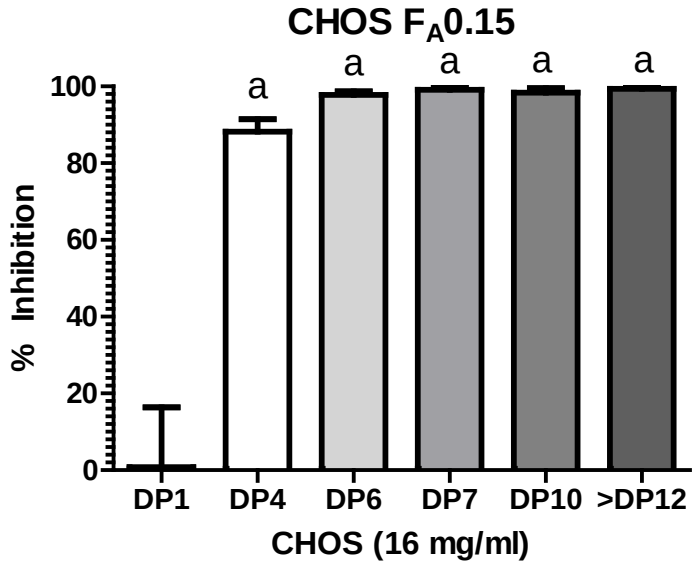

Fig.4. Inhibition of EPEC adherence to HEp-2 cells by purified CHOS fractions with different DP obtained by size-exclusion chromatography of enzymatically hydrolyzed F_A0.15 chitosan. Statistical analysis was performed by Analysis of Variance (ANOVA) to determine statistical differences from the control. Tukey's test was used to determine significant differences among the treatments. (n=4). Groups sharing the same letter are not significantly different from each other ($p < 0.05$).

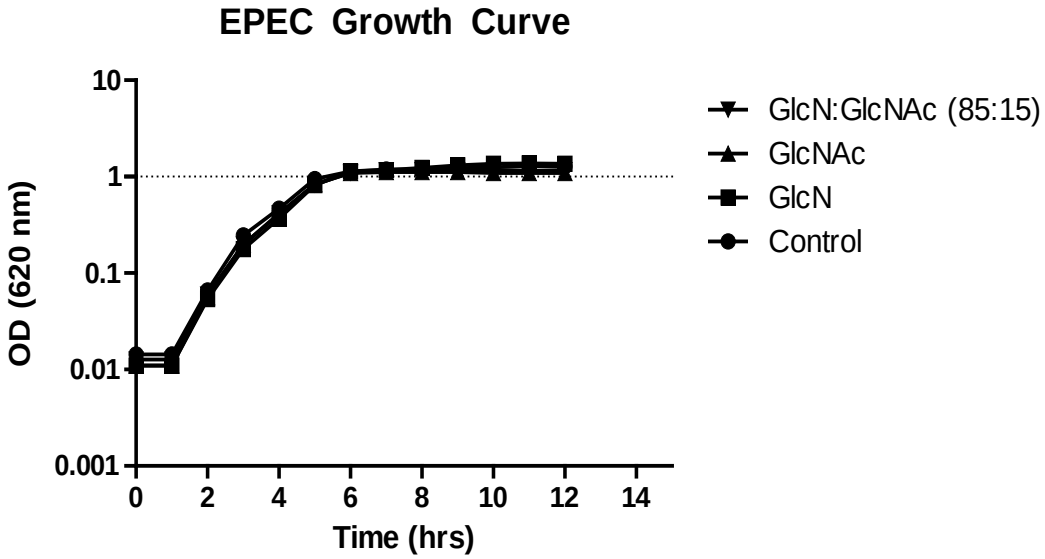

Figure 5 Growth of EPEC in the presence and absence of GlcN and GlcNAc. The graph shows the growth curve for EPEC in TSB at 37°C in the absence ("Control") or in the presence of added sugars. Sugars tested were GlcN, GlcNAc and a 85:15 mix of GlcN: GlcNAc, at a total sugar concentration of 16 mg/ml.

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