Carbohydrate Polymers 137 (2016) 617–624

Contents lists available at ScienceDirect

Carbohydrate Polymers

journal homepage: www.elsevier.com/locate/carbpol

Effect of chito-oligosaccharides over human faecal microbiota during fermentation in batch cultures

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ARTICLE INFO

Article history:
Received 2 July 2015
Received in revised form 4 November 2015
Accepted 5 November 2015
Available online 10 November 2015

Keywords:
Chito-oligosaccharides
Chitosan
Fermentation
Microbiota
Prebiotic

ABSTRACT

Chitosan with high number of deacetylated units, its reacetylated derivative and COS obtained through an enzymatic treatment with chitosanase were tested in pH controlled batch cultures to investigate the ability of the human faecal microbiota to utilise them.

Chitosan derivatives with high number of deacetylated units decreased the bacterial populations: *Bifidobacterium* spp., *Eubacterium rectale/Clostridium coccoides*, *C. Histolyticum and Bacteroides/Prevotella*. On the other hand, chitosan derivatives with high content of acetylated residues maintained the tested bacterial groups and could increase *Lactobacillus/Enterococcus*. Regarding short chain fatty acids (SCFA), only low M_w COS increased the production in similar levels than fructo-oligossacharides (FOS). The acetylated chitosans and their COS do not appear as potential prebiotics but did not affect negatively the faecal microbiota, while derivatives with high number of deacetylated units could induce a colonic microbiota imbalance.

1. Introduction

Chitosan is a natural biopolymer, a by-product of mainly crustacean waste from fishing industries. It is known to have important functional activities such as hydrating agent, film forming, preservative and flocculant (Aranaz et al., 2014) but its poor solubility makes it difficult to use in food and biomedical applications. Unlike chitosan, the low viscosity and good solubility of chito-oligosaccharides (COS) make them especially attractive for many uses such as mucoahesion, haemostatic, anti-inflammatory effects, antitumor activity, immunity enhancing and for food applications such as antioxidants, antimicrobials, antifungals and lipid binders (Aranaz et al., 2014; Muzzarelli, 2009; Xia, Liu, Zhang, & Chen, 2011). Depolymerization can be achieved through chemical or enzymatic methods. The last option is advantageous because the obtained COS keep their initial biological properties because of the mild conditions used and easy control of depolymerisation (Galed, Miralles, Paños, Santiago, & Heras, 2005; Kittur, Kumar, Varadaraj, & Tharanathan, 2005; Xie, Hu, Wei, & Hong, 2009).

Chitosan is approved as an additive in European Union (EU), although this approval is just for chitosan of fungal origin and just for wine-producing use (Regulation (EU) no 53/2011). Chitosan has antimicrobial activity and antioxidant activity that can improve the quality and useful shelf life of food (Kim & Rajapakse, 2005). It presents a wide bactericidal spectrum (Gerasimenko, Avdienko, Bannikova, Zueva, & Varlamov, 2004; Kim and Rajapakse, 2005; Kittur, Kumar, & Tharanathan, 2003; Kong, Chen, Xing, & Park, 2010; Tsai, Su, Chen, & Pan, 2002; Younes, Sellimi, Rinaudo, Jellouli, & Nasri, 2014). There are many dietary supplements with chitosan in the market because of its fat-binding capacity (Je & Kim, 2012; Muzzarelli, 1999; Santas, Espadaler, Mancebo, & Rafecas, 2012). Currently, the EU has authorized the claim about the maintenance of normal blood LDL-cholesterol concentrations that may be used only for food which provides a daily intake of 3 g of chitosan (Regulation (EU) no 432/2012).

The large intestine is a very complex ecosystem containing a large number of bacterial species. The environment is favourable for the growth of bacteria (beneficial and harmful) due to pH, high nutrients availability and the slow movements. Since the microbiota can result in compounds affecting to health, there is currently a great interest in knowing how different food ingredients can affect this ecosystem. The focus is overall in finding prebiotic ingredients (Corzo et al., 2015). Inulin and fructo-oligosaccharides (FOS) raises the growth of lactobacilli and bifidobacteria and decreasing Bacteroides and Clostridia (MacFarlane, Steed and Macfarlane, 2008)

Abbreviations: h, hour; min, minute; w/w, weight/weight; w/v, weight/volume; v/v, volume/volume; COS, chito-oligosaccharides; FOS, fructo-oligosaccharides.

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http://dx.doi.org/10.1016/j.carbpol.2015.11.011
0144-8617/

and they are the most studied and generally used as a positive control in the studies of new potential prebiotics. Only a limited number of studies have investigated the effect of chitosan and COS on the colonic microbiota. Lee, Park, Jung, and Shin (2002) showed that *Bifidobacterium bifidum* and *Lactobacillus spp*. could utilise COS in pure cultures. Li et al. (2007) and Liu et al. (2008) found an increase of *Lactobacillus* in visual counts of cultured cecum contents and faecal samples respectively, from broiler chicken and weaning pigs fed with COS. Wang, Yoo, Kim, Lee, and Kim (2009) showed also that visual counts of *Lactobacillus* on cultured faecal samples from growing pigs were not affected by COS supplementation. Recently, Kong et al. (2014) shows that COS increases some presumably beneficial bacterial species such as *Bifidobacterium spp*., *Bifidobacterium breve*, *Faecalibacterium prausnitzii*, *Lactobacillus* spp., *Prevotella*, *Fusobacterium prausnitzii*, *Methanobrevibacter smithii* and *Roseburia* and SCFA concentrations while decrease the amounts of several potential pathogens and concentrations of ammonia and branched chain fatty acids (BCFAs) in the intestinal luminal content of weaning piglets. Regarding studies about human microbiota, Vernazza, Gibson, and Rastall (2005) studied the *in vitro* fermentation of different commercial molecular weight chitosans and COS in human faecal cultures and did not observe an increase in bifidobacteria while potentially health-negative bacterial groups increased. Šimůnek, Koppová, Filip, Tishchenko, and Bełżecki (2010) showed measuring absorbance, that chitosan possessed a high antibacterial activity against *Roseburia intestinalis*, *Clostridium paraputrificum*, *Bacteroidesvulgatus*, *B. thetaiotaomicron*, *F. prausnitzii* and *Eubacterium lentum* isolated from human colon. Later, Šimůnek, Brandysová, Koppová, and Šimůnek (2012) studied measuring turbidity, that chitosan and low molecular weight chitosan (LMWC) inhibited the growth of six human origin bifidobacteria, however COS did not show any inhibitory effects. Therefore, chitosan effects on human intestinal microbiota should be studied further. Biofunctionality of chitosan and COS is closely related with their main physico-chemical characteristics, molecular weight (M_w) and acetylation degree (DA). The importance of carrying out the detailed physico-chemical characterization of COS is necessary to understand better the development of determined biological activities.

The aim of this study was to investigate the relationship between the physico-chemical characteristics (DA and M_w) of the obtained chitosan derivatives and the ability to be fermented by human faecal microbiota in batch cultures.

2. Materials and methods

2.1. Preparation of the chitosan parents

Chitosan parents were prepared from the commercial chitosan ChitoClear® fg 90 from fresh North Atlantic shrimp shells (*Pandalus borealis*) (Primex, Iceland). ChitoClear® fg 90 was purified by dissolving the chitosan in 0.5 M acetic acid. The solution was filtered through a series of 250–160 μm, 100–40 μm and 16–10 μm membranes to remove impurities in solution coming from chitosan production process.

After filtration chitosan was precipitated with 10% (v/v) NaOH. The precipitate was washed with EtOH:H_2O solutions (70:30, 80:20 90:10 and 100:0 proportions) until neutralization and dried at 50 °C. The purified chitosan obtained was one of the parents used (CHT A). The other parent chitosan (CHT B) was obtained by reacetylating CHT A. The reacetylation process consisted of dissolving CHT A in 0.5 M acetic acid at 1% (w/v) followed by 1,2-propanediol addition until 0.5% (w/v) chitosan concentration was reached. The stoichiometric addition of acetic anhydride taking into account the DA made possible reacetylation (Mengíbar, Mateos-Aparicio, Miralles, & Heras, 2013).

2.2. Preparation of the chitosan fractions

Chitosan fractions were obtained from CHT A and CHT B by enzymatic depolymerization with chitosanase (EC 3.2.1.132) from *Streptomyces griseus* (Sigma-Aldrich, St. Louis, MO, USA). The two chitosan parents were dissolved in 0.1 M acetate buffer at pH 5.7. Enzyme solution (3.48×10^{-3} mg/ml) was added to initiate the reaction and incubated at 37 °C in an orbital Lab Therm LT-Xshaker (Thermo Fisher Scientific Inc) at 100 rpm for four days. Ultrafiltration of the resuting samples was performed in Vivaflow Crossflow Cassettes connected to an air pressure controller with polyethersulfone membranes of 30, 10 and 5×10^3 g/mol cut-off pore sizes (Sartorius-Stedim Biotech, Germany). The ultrafiltration membranes isolated several fractions: $F > 30$, F 10–30, F 5–10 and $F < 5 \times 10^3$ g/mol. These fractions were dialysed in tubes with M_w cut-off 12–14, 7 and 3.5×10^3 g/mol pore size (Medicell International Ltd, London, UK) depending on obtained fraction until complete salt elimination, and afterwards lyophylized (Mengíbar et al., 2013).

2.3. Physicochemical characterization

^{13}C NMR was carried out in AMX 500 spectrophotometer (Bruker Ettinglen, Germany) for structural analysis in every obtained fraction. Samples were disolved in DCl/D_2O 1% (w/v) and TSP (3-(Trimethylsilyl) propionic-2,2,3,3-d_4 acid sodium salt) was used as internal standard. Acquisition time was 3 s with 17,000 scans. Acetylation degree (DA) was analysed by the first derivative UV-spectrophotometric method (Muzzarelli, Rocchetti, Stanic, & Weckx, 1997) and the weight average molecular weight (M_w) by SEC-HPLC was also determined.

2.4. In vitro *fermentation of the chitosan fractions and parent polymers*

The two parent polymers (CHT A and CHT B) and their corresponding derivatives (CHT A F 30, CHT A F 10–30, CHT A F 5–10, CHT A F 5, CHT B F 30, CHT B F 10–30, CHT B F 5–10, CHT B F 5) (Table 1) were evaluated for their effects on human faecal microbial populations and activity in 5 ml working volume pH controlled fermenters in parallel to fructo-oligosaccharides (FOS, BeneoP95, Orafti, Tienen, Belgium) as the positive control and faeces in basal media as negative control. Chitosan parents, chitosan fractions and FOS were used at 1% w/v in basal media. Basal medium ingredients (per litre) were: 2.0 g peptone water, 2.0 g yeast extract, 0.1 g NaCl, 0.04 g K_2HPO_4, 0.04 g KH_2PO_4, 0.01 g $MgSO_4.7H_2O$, 0.01 g $CaCl_2.6H_2O$; 2.0 g $NaHCO_3$, 2 ml Tween 80, 0.05 g haemin, 10 μl vitamin K_1, 0.5 g L-cysteine HCl, 0.5 g bile salts and 4 ml resazurin (0.05 g/l). Fermenter pH was maintained between 6.70 and 6.90, by means of a pH controller (Fermac 260, Electrolab, Tewkesbury,

Table 1
Average molecular weight (M_w) (g/mol) and acetylation degree (DA) values (%) for fractions of COS of CHT A and CHT B depolymerized with chitosanase determined by SEC and UV-spectroscopy, respectively.

Sample	M_w ($\times 10^3$ g/mol)	DA (%)
CHT A	180 ± 3.6	14 ± 0.8
CHT A F 30	28 ± 1.9	23 ± 3.5
CHT A F 10–30	18 ± 1.2	18 ± 1.6
CHT A F 5–10	9 ± 2.7	17 ± 1.6
CHT A F 5	3 ± 0.3	8 ± 0.7
CHT B	261 ± 8.5	35 ± 2.5
CHT B F 30	28 ± 1.3	47 ± 2.0
CHT B F 10–30	19 ± 1	46 ± 1.5
CHT B F 5–10	9 ± 0.7	38 ± 1.7
CHT B F 5	2 ± 0.05	37 ± 0.7

Values are means ± standard deviation ($n = 3$).