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The effect of preparation processes on the physicochemical characteristics and antibacterial activity of chitooligosaccharides

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ABSTRACT

The bioactivities of chitooligosaccharides are markedly influenced by the degree of acetylation, degree of polymerization or molecular weight and pattern of acetylation. Thus, it is crucial to identify reproducible processes that will give rise to well-defined chitooligosaccharides and establish methods for their posterior physicochemical characterization in order to advance in the knowledge of their bioactivity. Chitooligosaccharides were prepared by two different processes. The first used chitosanase enzymatic hydrolysis and the second consisted of a two-step procedure based on chemical hydrolysis followed by chitosanase hydrolysis. Chitooligosaccharides produced in the second process were composed of 63 % of fully deacetylated sequences and inhibited the growth of *Escherichia coli* and *Listeria monocytogenes*. Better antibacterial activity was found for those obtained in the first process composed of 27 % of fully deacetylated sequences. Therefore, a low percentage of free amino groups and the presence of acetylated sequences are necessary in these molecules to exert good antibacterial capacity.

1. Introduction

Chitooligosaccharides (COS) are linear co-oligomers of *N*-acetyl-D-glucosamine (A-units) and D-glucosamine (D-units) typically produced by partial hydrolysis from chitosan polymers. Chitosans are a versatile group of biopolymers with interesting physicochemical properties and promising biological functionalities (Aranaz et al., 2014). The biological activities of chitosan have been assumed to be at least in part due to the bioactivities of their enzymatic breakdown products generated by chitosan hydrolases present in the target tissues (Aam et al., 2010). Such partially acetylated chitosan oligosaccharides have been reported to possess, among others, anti-microbial activity, anti-inflammatory effects, fat-binding abilities, antitumor and immuno-stimulatory activities, and they are believed to accelerate absorption of calcium and iron *in vivo* (Kim & Rajapakse, 2005; Li, Xing, Liu, & Li, 2016; Ngo et al., 2015; Xia, Liu, Zhang, & Chen, 2011). However, the COS used in most studies were rather poorly characterized and frequently only average values for the molecular weights of the samples were provided, leaving room for doubt regarding the actual composition of the samples and, consequently, uncertainty as to the conclusions drawn concerning the bioactivity of chitooligosaccharides (Li et al., 2016). The structure-function relationship of COS bioactivities is thus still far from being fully understood (Chen, Zhu, Li, Guo, & Ling, 2010). The composition and bioactivity of a COS sample are closely related and will influence its physicochemical characteristics. Both are deeply dependent on the COS production process. The well-defined chitooligosaccharides available today are almost invariably mixtures of molecules differing in their degrees of either depolymerization (DP) or acetylation (DA), and even when these two parameters are clearly defined they will still be mixtures of molecules that could differ in their pattern of acetylation (Li et al., 2016).

Converting chitosan into chitooligosaccharides is an effective way to increase solubility and to decrease viscosity, enhancing the potential of these oligosaccharides as functional materials for many biological applications. Hence, numerous studies have focused on the partial chemical or enzymatic depolymerization of high molecular weight chitosan into COS (Song, Alnaeeli, & Park, 2014; Tang et al., 2010; Wu, Pan, Wang, & Wu, 2013). Chemical depolymerization typically involves acids such as HCl or HNO_2. These processes are well defined and can easily be performed on a large scale; their products are well known, but their actual composition is typically

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quite diverse (Vårum, Ottøy, & Smidsrød, 2001). Enzymatic depolymerization procedures, which have received a great deal of interest due to their environmental safety and easy control (Xia et al., 2011), yield slightly narrower variations in the mixtures of COS compared to chemical depolymerization. The structures of chitooligosaccharides obtained depend on the specificity of the enzyme used (Hsiao, Lin, Su, & Chiang, 2008; Lin, Lin, & Chen, 2009; Nidheesh, Kumar, & Suresh, 2015). The main drawback of enzymatic processes, aside from the high cost and limited availability of suitable enzymes, is their slow action on viscous polymer solutions. As a result, low substrate concentrations, and hence considerable volumes, are required, demanding rather large amounts of expensive enzymes.

We therefore chose to investigate a two-step process to obtain chitooligosaccharides, which consists of acid depolymerization followed by enzymatic hydrolysis. This procedure aims to combine the advantages of both processes and at the same time avoid their disadvantages. The idea is to first reduce the viscosity of the chitosan polymer substrate using the chemical step so that specific oligomeric products could then be generated in the enzymatic step. Naturally, the resulting COS will still be mixtures rather than pure oligomers, and the composition of the mixtures will depend on the acid and enzyme used. The reproducibility of the process will also be influenced by the polydispersity of the polymeric substrate. Still, it is important to keep in mind that obtaining such mixtures could be more efficient, in terms of both cost and yield, than the production of COS within a narrow range in degrees of depolymerization or even acetylation, which would involve a more complex purification process (Song et al., 2014). The goal is to define a reproducible process that reliably generates mixtures of COS with defined physicochemical properties and known biological performance.

One of the most often cited bioactivities of COS, with significant relevance for applications in the biomedical and food industries, is their antibacterial activity. Both polymeric chitosans and COS have been reported to possess antibacterial activity, and this has been shown to be dependent on DP (Mengíbar et al., 2011; Park, Daeschely, & Zhao, 2004) and DA (Takahashi, Imaia, Suzukia, & Sawai, 2008; Tsai, Su, Chen, & Pan, 2002; Kumar, Varadaraj, Gowda, & Tharanathan, 2007). Moreover, COS are thought to have advantages as antimicrobial agents and due to their higher solubility compared to chitosan they are presented as promising products in the prevention of microbial growth. However, when it comes to quantifying the activity, strain and mechanism proposed, the available literature on the antibacterial activities of COS is highly controversial in terms of protocol, and the differences in the COS used prevent direct comparisons between studies.

The aim of this study was to develop a reproducible protocol for the production of well-defined COS, either in a one-step enzymatic process or in a two-step acidic-enzymatic process, and then to characterize the mixtures obtained in terms of their structures and their antibacterial activities against gram negative *Escherichia coli* and gram positive *Listeria monocytogenes* strains.

2. Methodology

2.1. Preparation of COS

Chitosan (114 kDa; DA 16%) from shells of the spider crab *Lithodes antarcticus* (Infiqus S. L., Madrid, Spain) was dissolved (0.5%, w/w) in 0.5 M acetic acid and the solution was filtered through a series of glass Buchner funnels (250–160 μm; 100–40 μm and 16–10 μm) before use. The chitosan solution was precipitated with 10 % (w/v) NaOH aqueous solution and washed with successive ethanol:distilled water solutions (70:30; 80:20 and 90:10) to a final pH of 6.5 ± 0.2. Finally, the chitosan was dried at 50 °C and pulverized with an A505 2HF electric grinder (Moulinex, Spain). The powder was employed in the preparation of the subsequent processes.

In the one-step process (P1) chitosan was dissolved in 0.2 M acetic/acetate buffer at 0.5 % (w/v) (pH 5.7) and depolymerized using a commercial chitosanase from *Streptomyces griseus* (EC 3.2.1.132) (Sigma-Aldrich, St. Louis, MO, USA). One milliliter of enzyme (3.48×10^{-3} mg/ml) was employed per 100 ml of substrate and the reaction was carried out at 37 °C in an orbital Lab Therm LT-Xshaker (Thermo Fisher Scientific Inc.) at 100 rpm for 4 days (Mengíbar, Mateos-Aparicio, Miralles, & Heras, 2013). In the two-step process (P2), the same chitosan was dissolved in 0.1 M HCl at 0.68 % (w/w) and chemically hydrolyzed by adding 31.36 mM KNO_2 at 3.75 % (v/v) during 30 min at 37 °C in an orbital shaker at 100 rpm. The solution was precipitated by adding NH_4OH and washed with successive ethanol/distilled water solutions as described above. The resulting low molecular weight chitosan (LMWC) was depolymerized with the same chitosanase and under the same conditions described above for P1.

The resulting COS mixture was separated by tangential ultrafiltration using Vivaflow Crossflow Cassettes connected to a Vivaflow 200 system (Sartorius-Stedim Biotech, Germany) with 30, 10 and 5 kDa cut-off polyethersulfone membranes at the end of each process. Three different COS fractions were isolated from each process: P1-COS30-10 or P2-COS30-10 (M_w 30–10 kDa), P1-COS10-5 or P2-COS10-5 (M_w 10–5 kDa) and P1-COS < 5 or P2-COS < 5 (M_w <5 kDa). These fractions were dialyzed against distilled water in membranes with M_w 12–14, 7 and 3.5 kDa cut-off respectively (Medicell International Ltd, UK) until complete salt elimination. Dialyzed fractions were freeze-dried.

2.2. Physicochemical characterization of COS

Both processes were followed by HP-SEC in a Waters 625 LC System Pump with an Ultrahydrogel column (I.D.: 7.8 mm, L: 300 mm) thermostated at 35 °C and connected to a Waters 2424 evaporative light scattering detector (ELSD) (Waters Corp, Milford, USA). Samples were dissolved at 0.1% (w/v) in a 0.2 M acetic acid/0.15 M ammonium acetate buffer (pH 4.5) before injection and the same buffer was employed as eluent. The flow rate was 0.5 ml/min. The standard chitosan curve was used to determine the average molecular weight ($\bar{M}_w$) of the COS (Mengíbar et al., 2013).

^{1}H NMR spectra were recorded on a 300 MHz Bruker Advance Spectrometer (Bruker Ettinglen, Germany) for structural analysis in each purified fraction. Samples were dissolved at 0.5% (w/v) in DCl/D_2O 1% (Deutero GmbH, Germany) and acquisition time was 1 s with 32 scans. Measurements were recorded at 25 °C and DA was determined according to Hirai, Odani, & Nakajima (1991).

MALDI-TOF MS of the COS30-10 fractions was carried out using an Autoflex Speed MALDI-TOF mass spectrometer (Bruker Daltonics, Germany) equipped with a SmartBeam™ NdYAG-laser (355 nm) in positive ion mode and controlled using FlexControl v3.0. For ionization, 2,5-dihydroxybenzoic acid (10 mg/ml in acetonitrile:water (1:1)) was used as matrix. All spectra were obtained in reflection mode with an acceleration voltage of 25 kV, a reflector voltage of 26 kV, and pulsed ion extraction of 40 ns in positive ion mode. The acquisition range was m/z 50–4000. Previously, samples were dissolved at 0.1% (w/v) in 200 mM ammonium acetate buffer pH 4.2 and were depolymerized with a specific *endo*-chitosanase (CTSA) from *Alternaria alternata* (a donation from Dr. Moerschbacher's group, Westphalian Wilhelm's-University Münster, Germany). COS 30-10 hydrolysate was washed with sterile Milli-Q® water three times and the solvent was evaporated each time in a vacuum centrifuge (10 min, 18000 g at 4 °C). Supernatants were used for MALDI-TOF-MS analysis. The data were obtained by taking the average value of 500 laser shots, with the lowest laser