Antibacterial Activity of Chitooligosaccharides

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The aim of this study was to investigate the antibacterial activity of chitooligosaccharide, prepared by partial acid hydrolysis of chitosan, and of an aminoglycosylated derivative, prepared by reductive alkylation of the chitooligosaccharide, against *E. coli* and *S. aureus.*

Key words: Chitosan, Chitooligosaccharide, Aminoglycosylated Derivative

Introduction

Chitosan, a linear polymer composed of β-1,4-linked glucosamine with various degrees of *N*-acetylated residues, is a deacetylated derivative of chitin extracted from an abundant source of shellfish exoskeletons (Kittur *et al*., 2003). It is a weak base and insoluble in water and organic solvents (Kumar *et al*., 2004a). However, it is soluble in dilute aqueous acidic solution. Chitosan is inexpensive, biodegradable, and nontoxic. This makes it suitable for use as additive in the food industry, as hydrating agent in cosmetics, and more recently as pharmaceutical agent in biomedicine (Khor and Lim, 2003).

Related to antimicrobial activity, chitosan is superior to chitin since it contains many amino groups, which interact with the negatively charged residues of macromolecules at the surface of bacteria and subsequently inhibit bacterial growth (Kim *et al*., 2003). Low molecular weight chitosans (LMWC) with an average molecular weight in the range of 5,000–20,000 Da were shown to possess superior biological activities compared to chitosan (Muzzarelli, 2002).

The antimicrobial activity of chitosan has been recognized against some kinds of microorganisms; it is influenced by several factors such as degree of polymerization, degree of deacetylation and molecular weight. It is generally recognized that chitosan with a high degree of deacetylation has high antimicrobial activity (Jeon *et al*., 2001). However, chitosan showed antibacterial activity only in acidic medium, which is usually due to the poor solubility of chitosan at high pH values (Liu *et al.*, 2004).

It has been reported that oligomers of lower molecular weight than that of chitosan exhibit better biological activities than chitosan. Oligomers of chitosan like chitooligosaccharide could be easily prepared by acidic or by enzymatic partial hydrolysis of chitosan (Kim *et al*., 2003). It is also an important starting material for synthesizing biologically active oligosaccharide derivatives (Qin *et al*., 2006).

In the present study, we report the antibacterial activity of chitooligosaccharide, prepared by partial acid hydrolysis of chitosan, and of an aminoglycosylated derivative, prepared by reductive alkylation of the chitooligosaccharide.

Experimental

Materials

Chitosan and D-(+)-glucosamine hydrochloride were purchased from Sigma Chemical Co. (St. Louis, MO, USA). Sodium cyanoborohydride with reagent grade 95% was from Aldrich Chemical Co. (St. Louis, MO, USA).

General experimental procedures

FT-IR spectra of KBr pellets were recorded in the 4000–400 cm^{-1} region using a Shimadzu FT-IR 8400 instrument. Derivation, including Savitzky-Golay algorithm with 25 smoothing points, was performed using the OPUS/I.R. version 1.4 software incorporated into the hardware of the instrument (Lillo *et al*., 2007). Microanalysis was performed at the Facultad de Química, Universidad Católica de Chile, Santiago City, Chile.

Partial acid hydrolysis of chitosan

Chitosan (1.00 g) was heated for 1 h at 90 °C with 36 mL of 0.10 M HCl, cooled and poured into 100 mL of acetone. The precipitate was separated by centrifugation, washed three times with acetone, dissolved in water and freeze-dried.

Gel permeation chromatography

An aqueous solution of the partially hydrolyzed chitosan was chromatographed on a Sephadex G-75 column (100 mm × 13 mm) and eluted with 1% (v/v) acetic acid (pH 5.3). The column was calibrated with 2 mL of Blue Dextran 2000 (4 mg/mL) and D-glucose (4 mg/mL). Elution was monitored spectrophotometrically with the phenol-sulfuric acid reagent for sugars (Chaplin, 1986). The fractions collected were analyzed under UV light (480 nm) and the absorbance was graphed as function of the volume (each 5 mL) (Fig. 1).

Reductive alkylation

Partially hydrolyzed chitosan (0.4 g) was suspended in 20 mL of methanol/acetic acid (3:1 v/v) and 1.33 g of D-(+)-glucosamine hydrochloride in 15 mL of water, and 1.0 g of sodium cyanoborohydride was added. The mixture was stirred for 6 d at room temperature, filtered and the solid was washed exhaustively with methanol and dried to give a white powder, soluble in water (67% yield). Analysis: Calcd. (%) for $[(C_8H_{13}O_5N)_{0.3} \cdot (C_{18}H_{24}O_8N_2)_{0.43} \cdot (C_6H_{11}O_4N)_{0.27})]_n$: C 37.92, H 6.95, N 4.44; found (%): C 37.89, H 6.95, N 4.50.

Microorganisms

Standard strains of bacteria, namely *Escherichia coli* (ATCC 31705) and *Staphylococcus aureus* (ATCC 6538p), were used for determination of the antibacterial activity (Qin *et al.*, 2006).

Antibacterial activities

A series of tubes with average liquid that contains variable and well-known concentrations of the chitooligosaccharide and its derivative were prepared. Thereafter, each tube was inoculate with the microorganism and incubated at 37 °C for 18 h. The presence or absence of the development of microorganisms indicates the bacterial sensitivity to the compound tested. The lowest concentration that completely inhibits the bacterial growth was assigned as minimum inhibitory concentration (MIC) (Qin *et al.*, 2006).

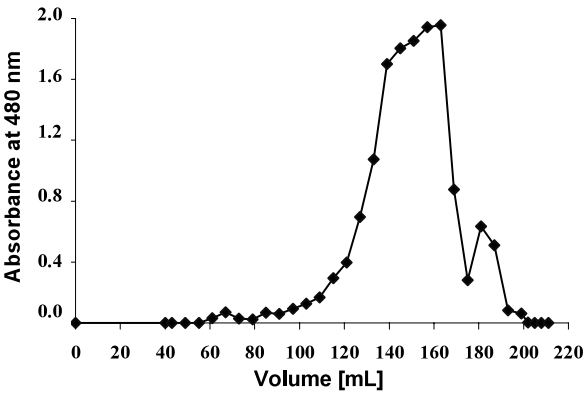

Fig. 1. Elution profile of partially hydrolyzed chitosan from Sephadex G-75 (each point corresponds to a collection of 5 mL, the first 40 mL correspond to the death volume).

Results and Discussion

The degree of *N*-acetylation of commercial chitosan determined by FT-IR spectroscopy according to Lillo and Matsuhiro (1997) was 30%. The main fraction (from 115 mL to 175 mL corresponding to a molecular weight between 1,000 to 10,000 Da) of the gel permeation chromatography on Sephadex G-75 of the with HCl partially hydrolyzed of chitosan gave a water-soluble compound with 56% yield (Fig. 1). The FT-IR spectrum (Fig. 2) showed characteristic absorption bands at 3423.38 cm^{-1} assigned to N–H and O–H stretching vibrations, at 2916.14 cm^{-1} assigned to C–H stretching vibration, at 1654.80 cm^{-1} assigned to C=O stretching vibration of the *N*-acetyl group and at 1596.94 cm^{-1} assigned to the N–H deformation vibration of a primary amine (Conley, 1966). The FT-IR spectrum of the main fraction of hydrolyzed chitosan was similar to that of the commercial chitosan. This evidence permits to infer that the basic structure of the polysaccharide was not affected.

Reductive alkylation (Fig. 3) of the amine group of chitooligosaccharide with D-(+)-glucosamine hydrochloride in the presence of sodium cyanoborohydride afforded the aminoglycosylated derivative with 67% yield. The derivative was analyzed by FT-IR spectroscopy and elemental microanalysis (Lim and Hudson, 2004). The FT-IR spectrum

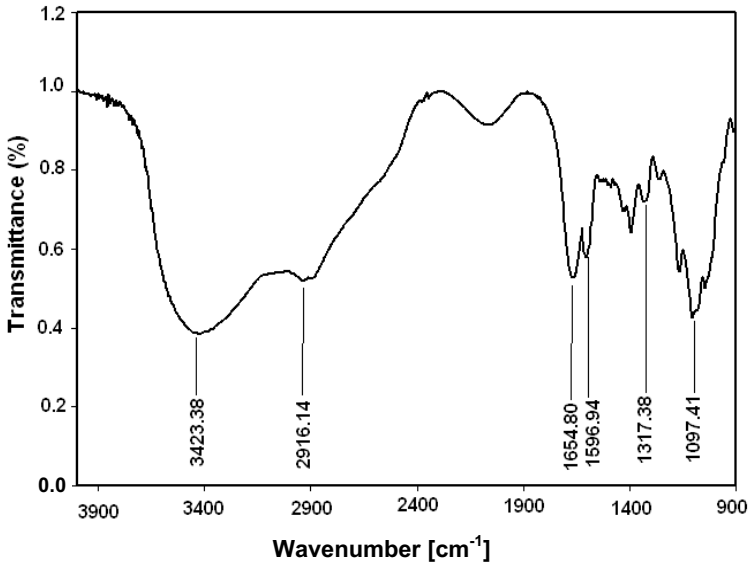

Fig. 2. FT-IR spectrum of the main fraction of gel permeation of partial acid hydrolysis of chitosan.

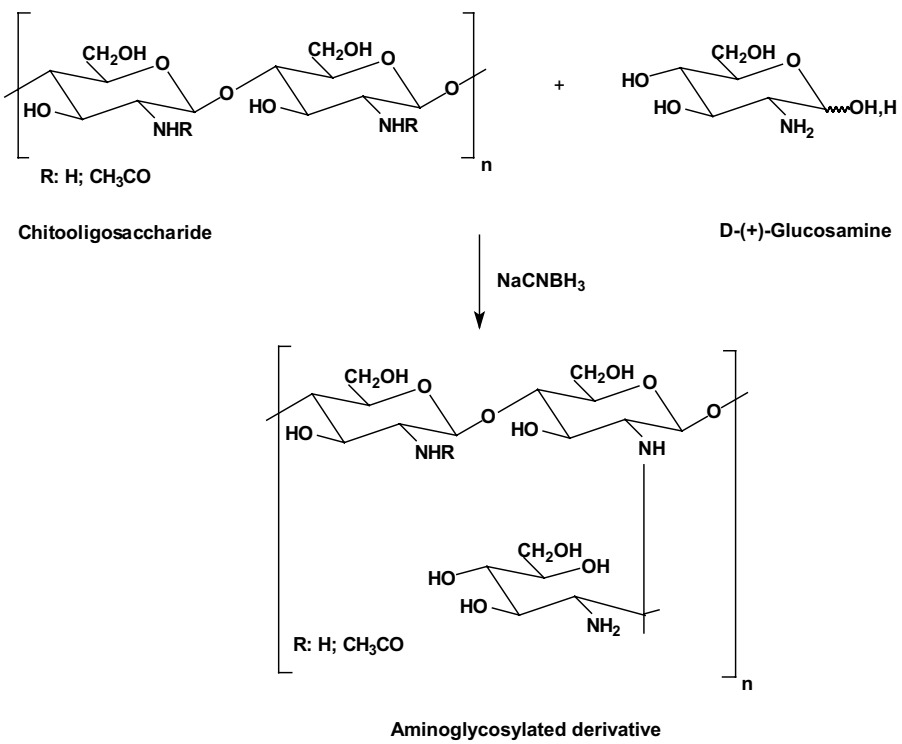

Fig. 3. Reductive alkylation of chitooligosaccharide to obtain the aminoglycosylated derivative.

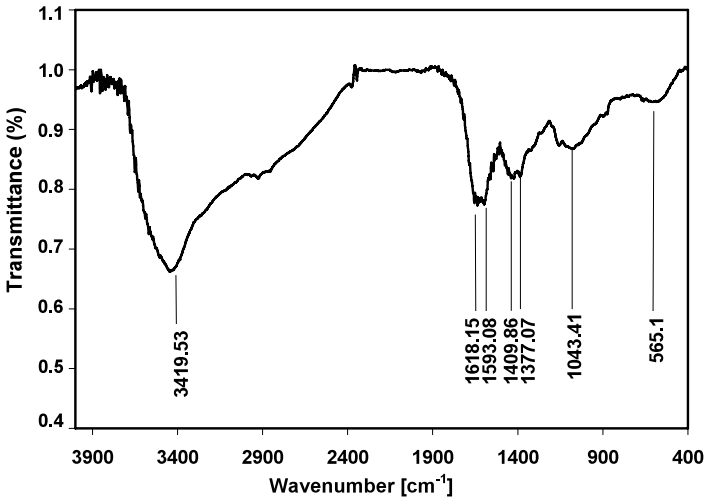

Fig. 4. FT-IR spectrum of the aminoglycosylated derivative of chitooligosaccharide.

(Fig. 4) of the derivative showed new bands at 1618.15 cm^{-1} assigned to the N–H distortion vibration of a secondary amine and at 565.1 cm^{-1} corresponding to the bending vibration of the $-NH_2$ group of the aminosugar introduced. Elemental microanalysis of the derivative indicated that approx. 40% of the free amino groups in the derivative are alkylated.

Our results showed that the main fraction from partial hydrolysis of chitosan has antibacterial activity. However, the structural modification by reductive alkylation of chitooligosaccharide did not increase the antibacterial activity.

The compounds assayed showed bacteriostatic activity with a MIC value of 1.13 and 1.06 μg/mL, respectively (Table I). The MIC and MBC values obtained for chitooligosaccharide were similar. Both compounds only showed activity against *S. aureus*.

The exact mechanism of antibacterial activity of chitosan still needs to be elucidated. Different mechanisms have been described. It may be due to staking of chitosan molecules on the microbial cell surface creating a polymer membrane, which blocks the transport of nutrients towards the cell (Kumar *et al.*, 2004b). Zheng and Zhu (2003) demostrated that chitosan weakened or even broke the cell membrane of *S. aureus*, impairing the physiological activities of the bacteria, and killed them.

Table I. Minimum inhibitory concentration of chitooligosaccharide and its aminoglycosylated derivate.

Compound	*E. coli*	*S. aureus*
Chitooligosaccharide	–	1.13 μg/mL*
Aminoglycosylated derivative	–	1.06 μg/mL*

* Values correspond to MIC. In the case of chitooligosaccharide, MIC and MBC are similar.

This study demostrates that the antibacterial activity of chitosan is effective. The product obtained by acid hydrolysis exhibits a similar behaviour as the compound obtained by enzymatic hydrolysis (Wang *et al.*, 2007). Additionally, our results demonstrate that the antibacterial activity could be correlated to the molecular weight, a result similar to that reported by Zheng and Zhu (2003).

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