© 2011 Boletín Latinoamericano y del Caribe de Plantas Medicinales y Aromáticas 10 (6): 536 - 542
ISSN 0717 7917
www.blacpma.usach.cl

Artículo Original | Original Article

Solubility effects on antibacterial activity of chemically modified chitooligosaccharides of fungal origin

[Efectos de la solubilidad sobre la actividad antibacteriana de quitooligosacáridos modificados químicamente de origen fúngico]

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Abstract
Chitin and chitosan are a class of metabolites that occurring in some fungi species that are associated with commercial and medicinal plants, this is in *Mucor* sp. for example with an ample number of biological activities, being antibacterial and antifungal one of the most important. Into our program of search of biopesticides and natural compounds with biological activities, we have studying chitosan that was obtained from the culture medium of the fungus *Mucor ruoxii*. Chitooligosaccharides were prepared by partial acid hydrolysis of native chitosan and an aminoglycosylated derivative was obtained by reductive amination of the chitooligosaccaride. The solubilities of these compounds were measured at different pHs and its antibacterial activity against *Escherichia coli* (gram-negative) and *Staphylococcus aureus* (gram-positive). Chitosan and the derivatives tested exhibited a good antibacterial activity against *S. aureus*.

Keywords: chitosan, *Mucor ruoxii*, chitooligosaccharide, antibacterial activity.

Resumen
Quitina y quitosano son una clase de metabolitos que producen algunas especies de hongos que están asociados con plantas medicinales y comerciales, esto es por ejemplo en *Mucor* sp., con un amplio número de actividades biológicas, siendo la antibacteriana y antifúngica unas de las más importantes. En nuestro programa de investigación de biopesticidas y compuestos naturales, estamos estudiando quitosano obtenido de el medio de cultivo del hongo *Mucor ruoxii*. Quitooligosacáridos fueron preparados por hidrólisis parcial ácida de quitosano nativo y un derivado aminoglicosilado fue obtenido por aminación reductiva del quitooligosacárido. Las solubilidades de estos compuestos fueron medidas a diferentes pHs y su actividad antibacteriana frente a *Escherichia coli* (gram-negative) and *Staphylococcus aureus* (gram-positive). Quitosano y los derivados testeados exhiben una buena actividad antibacteriana frente a *S. aureus*.

Palabras Clave: quitosano, *Mucor ruoxii*, quitooligosacárido, actividad antibacteriana.

Recibido | Received: August 23, 2011.
Aceptado en versión corregida | Accepted in revised form: September 28, 2011.
Publicado en línea | Published online: Novenber 30, 2011.
Declaración de intereses | Declaration of interests: We are grateful to Dirección de Investigación de la Universidad del Bío-Bío (Grant DIUBB 092309 1/R).
Este artículo puede ser citado como / This article must be cited as: Luis Lillo, Inés Muñoz, Jerónimo Perez, Julio Alarcón, Carlos L. Céspedes, Gerardo Cabello, Claudio Lamilla, Claudia Caro. 2011. Solubility effects on antibacterial activity of chemically modified chitooligosaccharides of fungal origin. Bol Latinoam Caribe Plant Med Aromat 10(6): 536 – 542.

INTRODUCTION

Chitin, a biopolysaccharide that occur naturally as a constituent of the cell wall of some fungi of the genera such as *Aspergillus* and *Mucor* (Qin et al., 2006). Chitosan, a linear polysaccharide of β (1→4) 2-amino-2-deoxy- D-glucan residues that is a derivative obtained by *N*-deacetylation of chitin (Kittur and Kumar, 2003).

Chitosan and its derivatives have been considered for various applications including flocculation and coagulation in food processing, heavy-metal ion recovery from wastewaters, and the fabrication of structural matrices for food, cosmetic, biotechnological and biomedical applications (Zúñiga *et al*., 2010; Yang *et al*., 2002). Chitosan is a weak base that is insoluble in water and organic solvents. However, it is soluble in dilute aqueous acidic media (Kumar *et al*., 2004). The poor solubility of chitosan becomes the major limiting factor for biological utilization. (Zhang *et al*., 2003; Khor and Lim, 2003).

Chitosan contains amino groups that interact with negatively charged residues of macromolecules on the surface of bacteria and subsequently inhibit bacterial growth (Kim *et al*., 2003). Low molecular weight chitosan (LMWC) with an average molecular weight in the range of 5,000 - 20,000 Da, possesses the most powerful biological activities (Muzzarelli *et al*., 2002).

Chitosan has shown antimicrobial activity against several kinds of microorganisms. This activity has been recognized and is fluenced by several factors such as degree of polymerization, degree of deacetylation and molecular weight (Jeon *et al*., 2001). However, chitosan showed antibacterial activity only in acidic media, which is probably due to the poor solubility of chitosan at high pH values (Liu *et al*., 2004).

It has been reported that chitoooligomers of lower molecular weight exhibit better biological activities than chitosan (Muzzarelli *et al*., 2002). Chitooligosaccharides, which can be easily prepared easily by acidic or by enzymatic partial hydrolysis of chitosan (Kim *et al*., 2003), provide an important starting material for synthesis biologically active derivatives (Qin *et al*., 2006).

The bioactivities evaluated in this article may provide novel insights into the functionality of chitosan and its derivatives, and potentially enable their use as functional-food components, additives and nutraceuticals (Mizuno *et al*., 1995; Jeon and Kim, 2002).

The aim of this study was to investigate the effects of pH on the solubility and its effect on antibacterial activity of chitosan obtained from *Mucor ruoxii*, and its derivatives prepared by partial acid hydrolysis and of an aminoglycosylated derivative prepared by reductive amination of the chitooligosaccharide against *E. coli* and *S. aureus*.

MATERIAL AND METHODS

Materials

Chitosan was obtained from extract of *Mucor ruoxii*, obtained from a culture maintained in our collection at the Departamento de Ciencias Básicas, Univerisdad del Bío-Bío, Chillán, Chile (Lillo *et al*., 2007a) and D-(+)-glucosamine hydrochloride was purchased from Sigma Chemical Co., (St. Louis, MO, USA). Sodium cyanoborohydride 95%, reagent grade, Aldrich Chemical Co., (St. Louis, MO, USA).

General experimental procedures

FT-IR spectra (KBr pellets) were recorded in the 4000 - 400 cm^{-1} region using a Shimadzu FT-IR 8400 instrument. Elemental analysis was performed at the Facultad de Química, Universidad Católica de Chile, Santiago, Chile.

Partial acid hydrolysis of chitosan

Chitosan (1.00 g) was heated for 1 h at 90º 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 (Lillo *et al*., 2007b).

Gel permeation chromatography

An aqueous solution of partially hydrolyzed chitosan was chromatographed on a Sephadex G-75 column (100 mm x 13 mm) and eluted with 1% v/v acetic acid (pH 5.3). Elution was monitored spectrophotometrically with the phenol-sulfuric acid reagent for sugars (Lillo and Matsuhiro, 2003).

Reductive amination

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 washed exhaustively with methanol and dried to give a white powder soluble in water (Guo *et al*., 2007).

Solubility determination

The solubility was determined dissolving 0.1 g of chitosan and derivatives, respectively in 10 ml of acetic acid (1% v/v). Sodium hydroxide (2 M) was added drop-wise to the solutions, while the pH was continually monitored until it became stable at pH 7.0 for 30 min (Yang *et al*., 2002).

Microorganisms

Standard strains of *Escherichia coli* (ATCC 31705) and *Staphylococcus aureus* (ATCC 6538p) were used for determination of antibacterial activity.

Antibacterial activities

A series of tubes containing different concentrations of the chitosan, of the oligosaccharide and of its aminoglycosylated derivative were prepared. Each tube was inoculated with the microorganism and incubated at 37º C for 18 h at pH 5.4. The presence or absence of the turbidity suggests the growth of microorganisms, which in turn indicates the bacterial sensitivity to the tested compounds. The lowest concentration that completely inhibited the bacterial growth was designated as the minimum inhibitory concentration (MIC) (Hu *et al*., 2007).

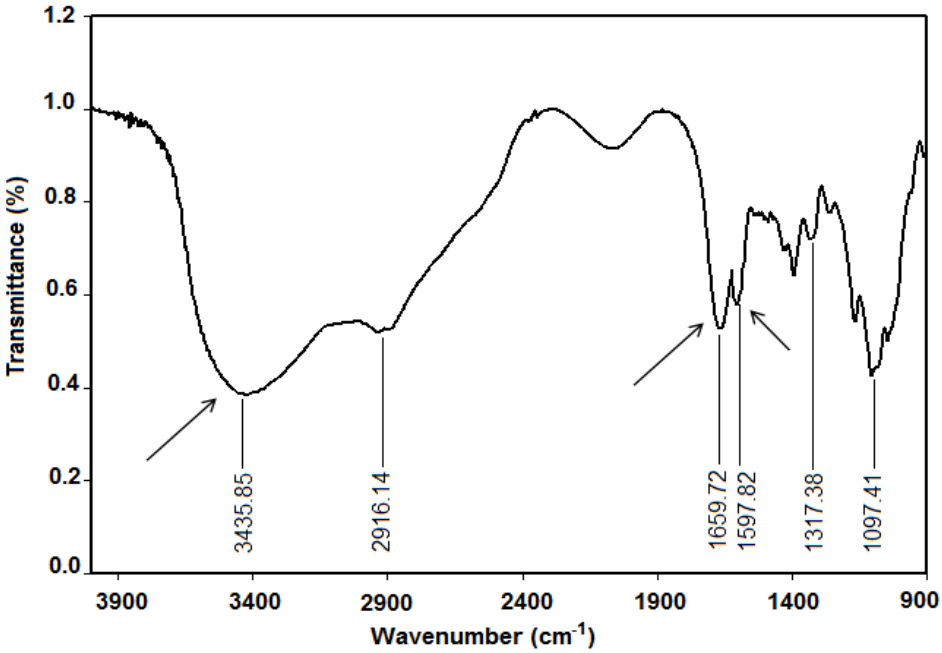

Figure 1
FT-IR spectrum of the main fraction of gel permeation of partial acid hydrolysis of chitosan showing the main signals.

RESULTS AND DISCUSSION

The degree of *N*-acetylation of chitosan obtained from fungi was determined by FT-IR spectroscopy according to Lillo and Matsuhiro (1997) and was 26%.

The main fraction of gel permeation chromatography on Sephadex G-75 of the partially hydrolyzed chitosan with HCl gave a water-soluble compound with 56% yield. The FT-IR spectrum (Figure 1) shows characteristic absorption bands at 3435.8 cm^{-1} assigned to N-H and O-H stretching vibration, at 1659.7 cm^{-1} assigned to C=O stretching vibration of the N-acetyl group and at 1597.8 cm^{-1} assigned to the N-H deformation vibration of a primary amine (Li *et al*., 2010). The FT-IR spectrum of the main fraction of hydrolyzed chitosan (17% *N*-acetylation degree) was similar to FT-IR of native chitosan. This evidence indicates that the basic structure of the polysaccharide was not affected.

The reductive amination reaction (Figure 2) of the amine group of the chitooligosaccharide with carbonyl moiety of D-(+)-glucosamine hydrochloride in the presence of sodium cyanoborohydride afforded the aminoglycosylated derivative in 67% yield.

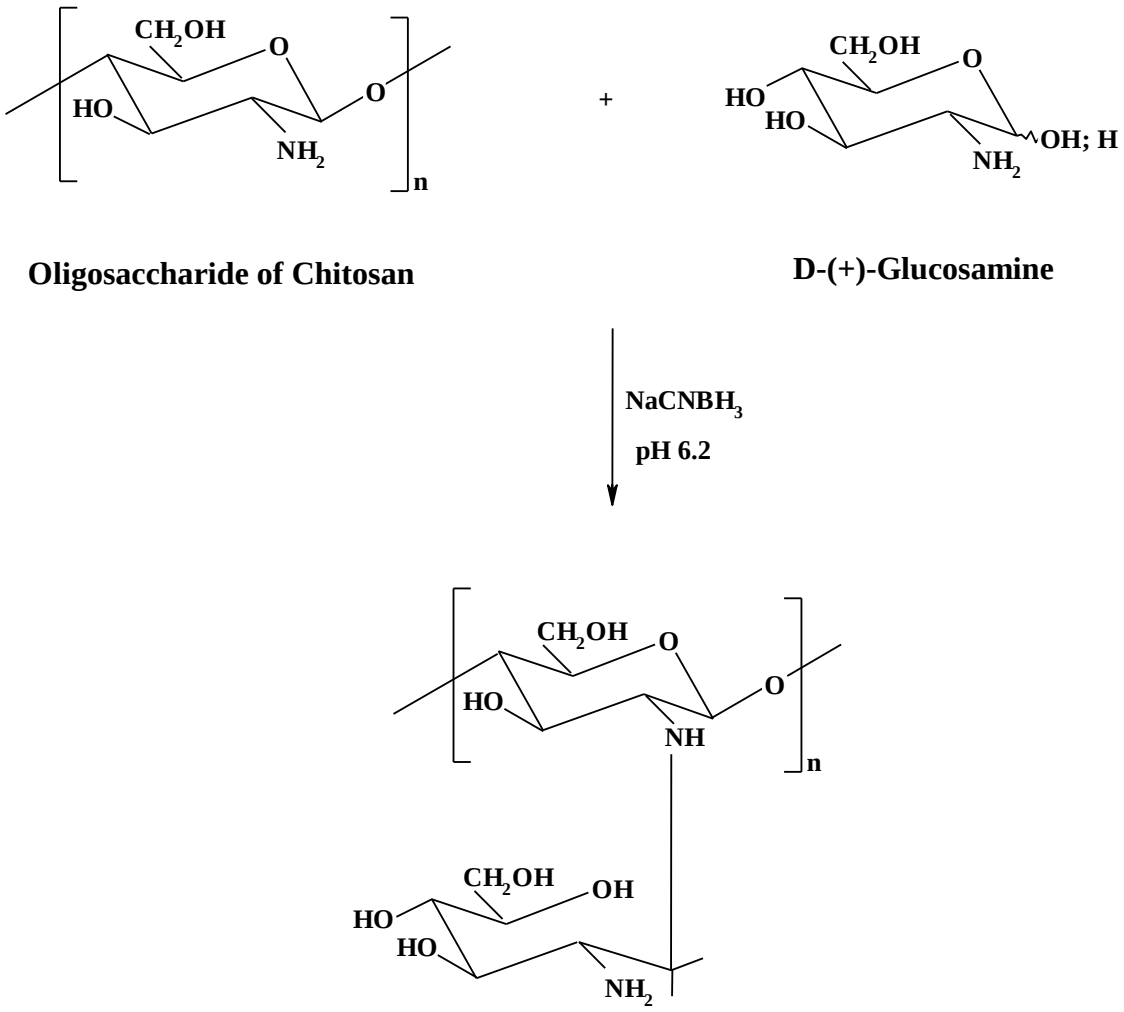

Figure 2
Reductive amination to obtain the aminoglycosylated derivative.

This derivative was analyzed by FT-IR spectroscopy and elemental analysis (Lim and Hudson, 2004). The FT-IR spectrum of the derivative (Figure 3) showed an increase in the intensity of the signal corresponding to hydroxyl group and the presence of new bands at 1620.2 cm^{-1} assigned to the N-H distortion vibration of a secondary amine and at 567.3 cm^{-1} corresponding to the bending vibration of the NH_2 group of the aminosugar introduced. Elemental analysis of the derivative indicated that the 40% of the free amino groups in the derivative were alkylated.

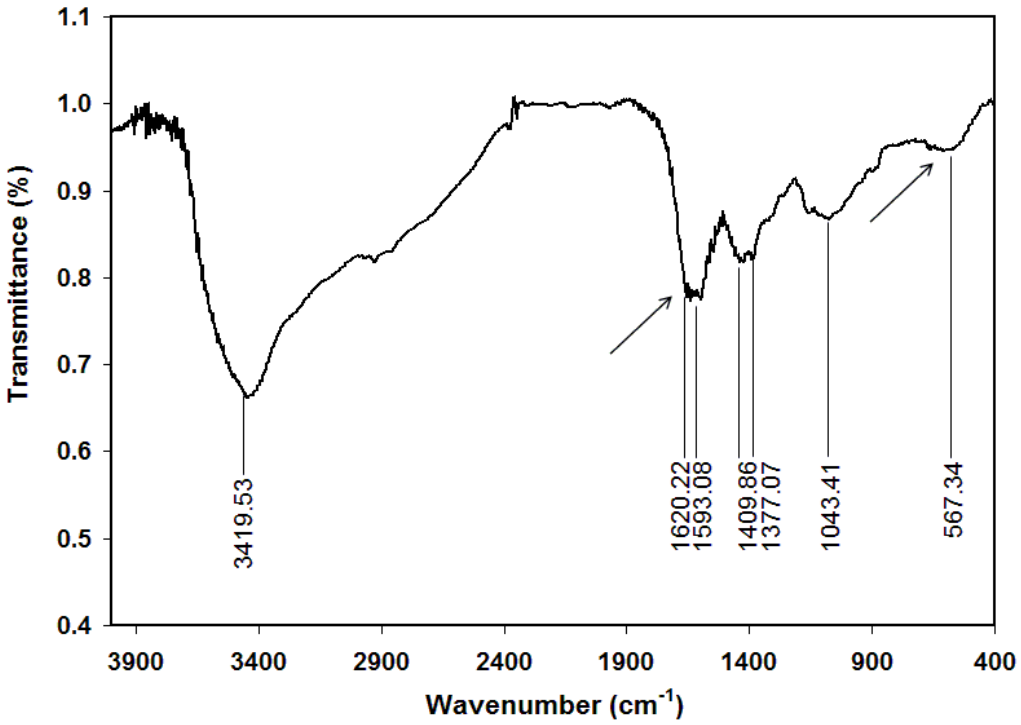

Figure 3
FT-IR spectrum aminoglycosylated derivative obtained by reductive amination showing the main signals.

Our results indicate that the chitosan and chitooligosacharide can be used to determine the antibacterial activity only at acidic pH (Table 1), whereas the aminoglycosilated derivative, being soluble, can be used in a wide range of pH values for the bioassays.

Table 1
Solubilities in [mg/ml] of chitosan and its derivatives*.

Compound	pH <6	pH 6-7	pH 7
Chitosan	22.3	n.s.**	n.s.**
Chitooligosaccharide	25.2	16.5	n.s.**
Aminoglycosylated derivative	28.7	20.4	19.2

*The pH of the solution was adjusted by the addition of 2 M aqueous NaOH. The concentration used was 10~30 (mg/ml).
**not soluble

The antibacterial activity of the compounds was evaluated at pH 5.4, and the MIC are shown in Table 2. At this pH all the compounds have the highest solubility.

Table 2
Minimum inhibitory concentration (MIC) in [μg/ml] of chitosan and derivates.

	Microorganism	
Compound	*E. coli*	*S. aureus*
Chitosan	-----	0.0102
Chitooligosaccharide	-----	1.13
Aminoglycosylated derivative	-----	1.06

This report demostrate that the antibacterial activity of chitosan is effective, but only can be assayed at pH 5.4. However, the derivatives can be assayed in water

over a wide pH range (pH ~ 5-7). The chitooligosaccharides obtained by enzymatic hydrolysis of chitosan, also exhibits a significant antimicrobial activity (Wang *et al*., 2007).

The exact mechanism of antibacterial activity of chitosan and its derivatives still needs to be elucidated. In Gram-positive bacteria the cell membrane is covered by a cell wall made up of 40 layers of peptidoglycans, which contain GLcNAc, *N*-acetyl muramic acid as well as D- and L-amino acids including teichoic acid (Hu *et al*., 2007). The positively charged amino groups of chitosan and its derivatives can bind to these, resulting in cell-wall distortion-disruption, exposure of cell membrane to osmotic shock and exudation of the cytoplasmic contents (Kumar *et al*., 2005).

On the other hand, Gram-negative bacteria contain an external membrane composed of lipopolysaccharide and proteins (Helander *et al*., 2003). The negatively charged O-specific antigenic oligosaccharide-repeating units of lipopolysaccharide form ionic bonds with the amino groups of chito-oligomers, thus blocking the flow of nutrients and causing concomitant bacterial death due to depletion of the nutrients. The molecular mass of the chitooligomers is an important factor that facilitates aggregation, which blocks nutrient flow and ultimately leads to cell lysis (Zheng and Zhu, 2003).

The assayed compounds only showed activity against *S. aureus* (Gram-positive), meanwhile *E. coli* (Gram-negative) was not affected probably due to molecular size of the compounds. We are working in the dissection of mechanism of the antibacterial action with the addition of commercial antibiotic in order to have an idea of the potency of chitosan and derivatives looking for the synergic effects.

CONCLUSIONS

This study demonstrates that the antibacterial activity of chitosan is effective, but is limited to acidic conditions due to its limited solubility above pH ~ 6.0, where chitosan starts to lose its cationic nature. Water solubility is important for chitosan to serve as an antimicrobial agent. Chitosan derivatives exhibit solubility in water over a wide pH range, and have excellent antibacterial activity against *S. aureus*.

ACKNOWLEDGEMENTS

We are grateful to Dirección de Investigación de la Universidad del Bío-Bío (Grant DIUBB 092309 1/R).

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