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Synthesis, characterization and antifungal activity of quaternary derivatives of chitosan on *Aspergillus flavus*

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ABSTRACT

Two series of new chitosan derivatives were synthesized by reaction of deacetylated chitosan (CH) with propyl (CH-Propyl) and pentyl (CH-Pentyl) trimethylammonium bromides to obtain derivatives with increasing degrees of substitution (DS). The derivatives were characterized by ^{1}H NMR and potentiometric titration techniques and their antifungal activities on the mycelial growth of *Aspergillus flavus* were investigated *in vitro*. The antifungal activities increase with DS and the more substituted derivatives of both series, CH-Propyl and CH-Pentyl, exhibited antifungal activities respectively three and six times higher than those obtained with commercial and deacetylated chitosan. The minimum inhibitory concentrations (MIC) were evaluated at 24, 48 and 72 h by varying the polymer concentration from 0.5 to 16 g/L and the results showed that the quaternary derivatives inhibited the fungus growth at polymer concentrations four times lower than that obtained with deacetylated chitosan (CH). The chitosans modified with pentyltrimethylammonium bromide exhibited higher activity and results are discussed taking into account the degree of substitution (DS).

1. Introduction

Chitosan is a polysaccharide usually obtained from deacetylation of chitin, which after cellulose is the second most abundant natural biopolymer found in nature. It may be extracted from various sources, particularly from exoskeletons of arthropods of crustaceans, fungi, insects, annelids, mollusks and coelenterata. The structures of chitin and chitosan correspond to those of poly[β(1→4)-2-acetamide-2-deoxy-D-glucopyranose] and poly[β(1→4)-2-amino-2-deoxy-D-glucopyranose], respectively (Ravi Kumar 2000). The homopolymer is a weak base with a pK_a value of the D-glucosamine residue of about 6.2–7.0 and is therefore insoluble at neutral and alkaline pH values. In acidic mediums, the amine groups will be positively charged, conferring to the polysaccharide a high charge density. Due to its polycationic nature, chitosan, after being dissolved in aqueous acid solutions, can be easily molded and used as membranes, beads, microparticles and gels (Rinaudo 2006; Ravi Kumar 2000). Also, its functional properties such as biodegradability and low toxicity (Domard 2011; Kean and Thanou 2010) have driven the research and applications of chitosan to medicine (Rinaudo 2006; Shi et al. 2006; Jayakumar et al. 2010), foods additives and preservatives (Shahidi et al. 1999) as well as in the paper industry and for the treatment of industrial wastewater (Ngah and Teong 2011).

One of the most attractive features of chitosan is its antibacterial, antiviral and antifungal activity (Zhang et al. 2011; Jayakumar et al. 2011). Recently the utilization of chitosan as a food preservative or adjuvant in agriculture to protect or stimulate the defense of different crops has increased (Zhang et al. 2011; Jayakumar et al. 2011). It is well known that pesticide residues are toxic for humans and animals and many of them are not biodegradable, what can cause serious environmental problems such as contamination of the water and soil (Satpathy et al. 2011). Chitosan and its oligomers have emerged as a promising source for many applications since it can be used to produce biodegradable fungicides to regulate the growing of plants and to protect seeds (Alburquenque et al. 2010).

The antifungal activity of chitosan is believed to occur from the interaction between the cationic chain and the negatively charged residues of macromolecules exposed on the fungal cell surface, leading to leakage of intracellular electrolytes and other constituents (Muzzarelli et al. 2001). It is believed that chitosan may affect the morphogenisis of the cell wall interfering directly on the activity of enzymes responsible for the growing of the fungi (El Ghaouth et al. 1992). Recently Li et al., based on confocal laser

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scanning microscopy of fluorescein-labeled chitosans, showed that low molecular weight chitosans could enter into the hypha of *Fulvia fulva*, suggesting that the growth of *F. fulva* could be inhibited by chitosan from the inside of the cell (Li et al. 2011).

The fungus *Aspergillus flavus* is considered one of the most serious problems in the production and consumption of grains in the world as it is related to the production of mycotoxins, such as aflatoxins, which are produced as secondary metabolites by some strains of *A. flavus* (Binder et al. 2007; Zhang et al. 2009). Fajardo et al. (1994) reported that chitosan limited *A. flavus* growth and subsequent aflatoxin production by inducing susceptible tissues to produce more phenolic compounds. Recently, Zhang et al. prepared chitosan-based blend films using chitosan, soybean trypsin inhibitor extracts and glycerol solutions (Zhang et al., 2009). The authors showed that the germination and growth of *A. flavus* were strongly inhibited by films prepared from soybean trypsin inhibitor extract (STI)/wild soybean trypsin inhibitor extract (WTI) and glycerol (Gly) solutions (chitosan-STI/WTI-Gly), indicating that the films could be useful as potential bio-control packaging against *A. flavus* during the peanut's and other cereal's storage. The study of new biomolecules having antifungal activities has emerged as a new subject and the modification of the chitosan structure aiming to improve its activity is a promising way to achieve effective biofungicides (Kenawy et al. 2005; Másson et al. 2008) and bactericides (Xu et al. 2011).

The aim of the paper is to report a simple and reliable method to prepare chitosans derivatives with improved capabilities of inhibiting the growth of *A. flavus*. The synthesis, characterization and antifungal activity of chitosan derivatives against the fungus *A. flavus* are described. A series of chitosan derivatives was obtained by the reaction of the amino groups of chitosan with propyl and pentyltrimethyl ammonium bromides. The results of the antifungal activity of these derivatives were presented and discussed, taking into account the degree of substitution of the substituted derivatives.

2. Materials and methods

2.1. Materials

Chitosan (degree of deacetylation (DD) 85%) was purchased from Polymar Co., Brazil, (3-bromopropyl) trimethylammonium bromide, (5-bromopentyl)trimethylammonium bromide, sodium hydroxide, sodium acetate, and acetic acid were purchased from Sigma Aldrich Chemical Co., Brazil. Spectra/pore membranes (Spectrum) were employed for dialysis. All solvents were of reagent grade and used as received. Water was deionized using a Gehaka water purification system.

2.2. Instrumentation

^{1}H NMR spectra were recorded on a Bruker ARX-500 500 MHz spectrometer. UV/Vis spectra were measured with a Cary 100 spectrophotometer equipped with a Peltier system. pH values of the solutions were determined using an Digimed pH-meter.

2.3. Preparation of the alkyltrimethylammonium-modified chitosans

Chitosan was deacetylated as described earlier (Tiera et al. 2006). The resulting polymer was purified by dialysis against water for 3 days and isolated by lyophilization. Next, chitosans with varying amount of grafted alkyltrimethylammonium bromides were prepared in aqueous NaOH solutions and the procedure is described below (Fig. 1). The degree of substitution was varied by setting the initial molar ratio as described in Table 1. A suspension of deacetylated chitosan (1.5 g, 9.3 mmol) in aqueous (20 mL) was prepared and the pH was adjusted to 9.0 by adding NaOH 0.1 M from an adapted funnel to a round-bottomed reaction flask equipped with a magnetic stirrer. Further (3-bromopropyl) trimethylammonium bromide (1.0 g) dissolved in water (20 mL) was added with stirring. Stirring was continued at 60 °C for 72 h and the pH was continuously monitored during the reaction time. The mixture was then dialyzed (membrane of MWCO 12–14,000 g/mol) to remove the unreacted alquiltrimethylammonium bromide, first against water for 2 days, then against aqueous NaOH (0.05 M) for 1 day, and finally against water for 2 days. The product was isolated by lyophilization and characterized by ^{1}H NMR and potentiometry.

2.4. Viscosity measurements

Viscosity measurements were carried out in water thermostated bath with a capillary calibrated viscosimeter for dilution Cannon-Ubbelohde 9722M-50 (Cannon Instr. Co.) at pH 4.5 acetic acid (0.3 M)/sodium acetate (0.2 M) buffer. The viscosimeter was immersed in a thermostatic bath at 298.15 ± 0.05 °C and the samples were allowed to equilibrate for 10 min in the bath before measurements. Measurements at each concentration were repeated and the reproducibility was better than ±0.01 s. The results of the viscosity measurements were expressed as reduced viscosity values calculated from

$$\eta = \frac{(t - t_o)/t_o}{c}$$

where t is the measured efflux time of the polymer solution, t_o is the efflux time of the pure solvent, and c is the polymer concentration (g/L). The mean viscosimetric molecular weight of chitosan and its derivatives were determined by using the Mark–Houwink equation, with constants $a = 0.796$, $K = 0.079\ \mathrm{mL\,g^{-1}}$ (Roberts and Wang 1996).

2.5. Pathogen and cultures

The microorganism chosen to test the antifungal activity of chitosan and its derivatives was *A. flavus*. The strain was kindly provided by Brazilian Collection of Microorganisms from the Environment and Industry – CBMAI, Campinas – São Paulo, Brazil, and it was maintained on potato dextrose agar (PDA) (potato infusion from 200 g/L, 20 g/L dextrose, and 15 g/L agar) in the dark at 25 ± 2 °C.

2.6. Antifungal assays

The antifungal activity of deacetylated and commercial chitosan was compared to those of the alkyltrimethylammonium derivatives modified with a degree of substitution varying from 0.5 to 65%. The polymers solution were prepared at pH 5.5 in acetic acid and added at concentrations of 0.0 (control plate) 0.1, 0.5 and 1.0 g/L to the melted culture medium, which contained 10.0 mL of 10% potato dextrose broth and then transferred to Petri dishes. After solidification, the mixtures were inoculated with a 1 mm in diameter mycelium fungus *A. flavus* at the center of Petri dishes and these were incubated in an oven for 7 days at 25 ± 2 °C. Inhibition index of the fungus by the polymers was determined by the radial growth of the colony with a caliper on the 3rd, 5th and 7th days of cultivation, with the result of the 7th day used for comparative purposes.

The antifungal index was calculated as follows:

$$\text{Antifungal index} \quad (\%) = \frac{1 - D_a}{D_b} \times 100,$$