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Short communication

Evaluation of the antimicrobial activity of chitosan and its quaternized derivative on *E. coli* and *S. aureus* growth

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ABSTRACT

Chitosan is largely known for its activity against a wide range of microorganisms, in which the most acceptable antimicrobial mechanism is found to include the presence of charged groups in the polymer backbone and their ionic interactions with bacteria wall constituents. This interaction suggests the occurrence of a hydrolysis of the peptidoglycans in the microorganism wall, provoking the leakage of intracellular electrolytes, leading the microorganism to death. The charges present in chitosan chains are generated by protonation of amino groups when in acid medium or they may be introduced via structural modification. This latter can be achieved by a methylation reaction resulting in a quaternized derivative with a higher polymeric charge density. Since the charges in this derivative are permanents, it is expected a most efficient antimicrobial activity. Hence, in the present study, commercial chitosan underwent quaternization processes and both (mother polymer and derivative) were evaluated, in gel form, against *Staphylococcus aureus* (Gram-positive) and *Escherichia coli* (Gram-negative), as model bacteria. The results, as acquired from turbidity measurements, differ between materials with an expressive reduction on the Gram-positive microorganism (*S. aureus*) growth, while *E. coli* (Gram-negative) strain was less sensitive to both polymers. Additionally, the antibacterial effectiveness of chitosan was strongly dependent on the concentration, what is discussed in terms of spatial polymer conformation.

Introduction

Chitosan is a natural unbranched homopolymer obtained from chitin, an abundant by-product of seafood processing, via a deacetylation reaction (removal of acetyl groups $COCH_3$ from the chitin original structure) with alkali (Kurita, 2006). The final chitosan structure has one primary amine and two free hydroxyl groups for each monomer and can be expressed by the general formula $C_6H_{11}O_4N$. Chitosan has good film-forming ability and due to its high versatility, this polymer has been extensively evaluated for uses in food conservation (Britto and Assis, 2007a), in biomedical applications (Singh and Ray, 2000), as material for chemicals encapsulation and controlled release (Patel and Jivani, 2009) and in environmental remediation (Assis and Britto, 2008).

Commercially, chitosan is found from a variety of sources such as crabs, shrimp, lobster etc., usually sold in powder or as flakes form. The molecular weight and the degree of deacetylation are the main parameters which defines solubility and physic-chemical properties of this polymer. To be transformed in films or pieces, the chitosan should be first solubilized into gel by appropriate solvent dissolution. Crude chitosan however, is only soluble in acid medium, in pH below its pK_a (around 6.4). Such means a drawback for broader applications of chitosan, as the pH plays an important role on its biocompatibility (Kurita, 2006) and on film mechanical properties (Britto et al., 2005).

When chitosan molecules are submitted to an intensive methylation process, a derivative salt with permanent positive charges is generated as consequence of the quaternization of the amino groups (identified as trimethylchitosan – TMC) (Britto and Assis, 2007b). The presence of these charges in the polymer backbone gives to chitosan a cationic characteristic independent of the solvent pH. TMC can be prepared into gel in neutral medium and therefore, better suited, mainly for food and medical applications (Singh and Ray, 2000; Ji et al., 2009).

Additionally several models suggested that the antimicrobial activity of chitosan is a result from its cationic nature (Rabea et al., 2003; Goy et al., 2009). The electrostatic interaction between positively charged $R{-}N(CH_3)_3^+$ sites and negatively charged microbial cell membranes, is predicted to be responsible for cellular lysis and assumed as the main antimicrobial mechanism (Rabea et al., 2003;

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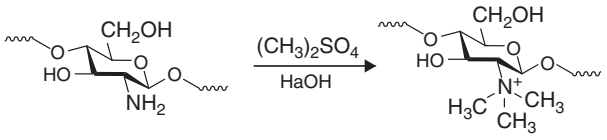

Fig. 1. Schematic representation of the reaction leading to the quaternization of the amino groups of chitosan resulting in *N,N,N*-trimethylchitosan (TMC). Quaternization experimental details and TMC characterization and can be found in Britto and Assis (2007a,b).

Tripathi et al., 2008). Charged chitosan can also interact with essential nutrients therefore interfering on microbial growth (Jia et al., 2001). Consequently is expected that polymers with higher charge densities resulted in an improved antimicrobial activity.

In the present study, commercial chitosan was used as precursor for transformation into charged derivative TMC and both materials, in gel form, was evaluated as antimicrobial agent against the Gram-negative bacterium *E. coli* and the Gram-positive *S. aureus* (common foodborne and hospital-acquired pathogen) as a function of polymer concentration.

Materials and methods

Methylation process

The starting chitosan was of medium molecular weight (400,000 g/mol, 75–85% unities deacetylated – shrimp origin) purchased from Sigma-Aldrich Co. (St. Louis, MO, USA) and used as supplied. For the methylation reaction, a developed methodology patented by Embrapa (Empresa Brasileira de Pesquisa Agropecuária) (Britto and Assis, 2007c), was used. In brief the reaction consists in the addition of 1.2 g of NaOH (0.015 mol) plus 0.88 g of NaCl (0.015 mol) in a suspension of 1 g of chitosan (0.005 mol) in 16 ml of dimethylsulfate (Synth, R. Janeiro, Brazil) and 4 ml of deionized water. The mixture was stirred and the derivative obtained by precipitation with acetone was rinsed and vacuum dried.

The methylation process results in the quaternized derivative TMC (*N,N,N*-trimethylchitosan), by inserting methyl functionality onto chitosan amino groups at the C-2 position (Fig. 1). Methylation details and a full characterization of TMC structure can be found elsewhere (Britto and Assis, 2007a,b).

Gel forming

Gels were prepared by dissolving the chitosan in 1% acetic acid (pH 4.0) in deionized water and the TMC solubilized directly in distillated water (pH 6.6). The gels were homogenized for 2 h under moderated magnetic stirring. Polymer concentrations of 0.5, 1.0, 1.5 and 2.0 g/l were prepared for each material.

Inoculums preparation

Escherichia coli (ATCC 8739) and *Staphylococcus aureus* (ATCC 25923) both provided by Fundação Tropical André Tosello, Campinas, Brazil, were used as bacteria models to evaluate the activity of parent and derivate polymer. The bacteria pre-culture was incubated under aerobiosis and moderate shaking for 24 h. The *E. coli* was kept at 37 °C and the *S. aureus* at 32 °C, considering the ideal temperature for each colony growth (Aneja et al., 2009). The bacterial kinetic was determined by measuring the absorbance at 620 nm wavelength hourly, following Bohinc et al. (2015) procedure, using a Shimadzu UVPC 2000 (Shimadzu Co. Kyoto, Japan) spectrophotometer. The stationary phase was taken as a reference time for comparing the polymeric effect on bacterial growth.

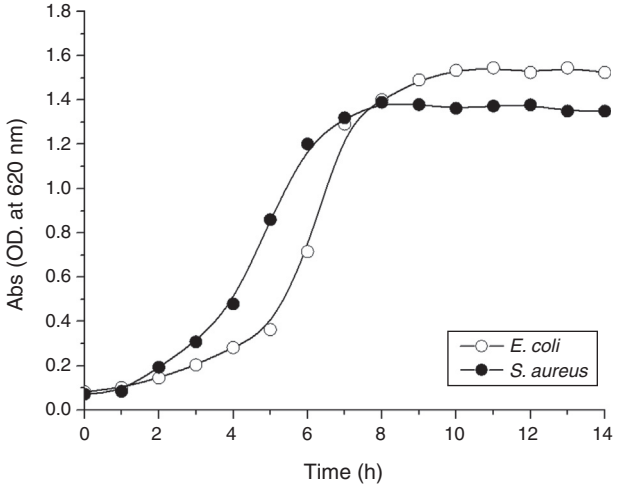

Fig. 2. Growth profile for *E. coli* (Gram-negative) and *S. aureus* (Gram-positive) as assayed by turbidity method at 620 nm.

Antibacterial assay

The inhibitory effects of chitosan and TMC on the bacterial growth were first estimated by means of turbidity measurements. We took 2×10^8 bacteria/ml as a reference for initial colony quantification (Koch, 1994). To attain this figure, sequential dilution was necessary (six for *E. coli* and four for *S. aureus*) according to simultaneous counting of plate colonies (CFU). For antimicrobial analysis, aliquots of 1 ml of bacterial broth were added to 9 ml of chitosan diluted suspensions and kept under moderate shaking at room temperature. The turbidity was measured in each polymeric sample solutions by adding to the mixture of the cultured bacteria medium and PBS (Phosphate-buffered saline), pH 7.4.

The plate well diffusion method (Rayn et al., 1996), was also used to visualize the formation of a zone of inhibition in a TSB (tryptic soy broth) solid culture medium. The procedure carried used in this analysis follows the agar diffusion method according to Dutta et al. (2009) procedure, in which small circular cavities are punctured in the culture medium and filled with approximately 0.25 ml of gels for each polymer concentration. 50 μl of bacterial suspension were spread and the plates stored for 24 h at 32–37 °C to allow microorganism growth. Inhibition zones were measured on bases of the average diameter of the clear area, directly on the dishes. Three replicate plates were used for each concentration and data were subjected to statistical evaluation by one-way analysis of variance (ANOVA). The significance $p \leq 0.05$ was considered using a Microcal Origin 9.0 software (OriginLab Co., Northampton, MA, USA).

Results and discussion

The growth kinetics curves of *E. coli* and *S. aureus*, as measured by turbidity at 620 nm, is presented in Fig. 2, where OD stands for *Optical Density*. Both bacteria grow in a similar way but with differences in turbidity. It is recorded an exponential increasing (log phase) during the first 6–8 h, followed by the stable stationary phase. The log phase for *E. coli* appears to be longer than that measured for *S. aureus*. The kinetics curves for both bacteria are in complete agreement to several examples found in the literature (Duffy et al., 1999; Fujikawa and Morozumi, 2006). The turbidity readings with the polymer addition were then carried out after 12 h incubation, assuring the attainment of maximum microorganisms per volume (plateau).

When the polymeric medium is mixed with the referential broth, the growth rate is temporarily affected, causing a reduction