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Influence of chitosan and its derivatives on cell development and physiology of *Ustilago maydis*

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ABSTRACT

Ustilago maydis, a dimorphic fungus causing corn smut disease, serves as an excellent model to study different aspects of cell development. This study shows the influence of chitosan, oligochitosan and glycol chitosan on cell growth and physiology of *U. maydis*. These biological macromolecules affected the cell growth of *U. maydis*. In particular, it was found that chitosan completely inhibited *U. maydis* growth at 1 mg/mL concentration. Microscopic studies revealed swellings on the surface of the cells treated with the polymers, and chitosan caused complete destruction of the membrane and formation of vesicles on the periphery of the cell. Oligochitosan and chitosan caused changes in oxygen consumption, K^+ efflux and H^+-ATPase activity. Oligochitosan induced a faster consumption of oxygen in the cells, while glycol chitosan provoked slower oxygen consumption. It is noteworthy that chitosan completely inhibited the fungal respiratory activity. The strongest effects were exhibited by chitosan in all evaluated aspects. These findings showed high sensitivity of *U. maydis* to chitosan and provided evidence for antifungal effects of chitosan derivatives. To our knowledge, this is a first report showing that chitosan and its derivatives affect the cell morphology and physiological processes in *U. maydis*.

1. Introduction

Chitosan (CH), a linear polymer derived by deacetylation of chitin, is formed by β-(1,4)-2-acetamido-2-deoxy-D-glucose and β-(1,4)-amino-2-deoxy-D-glucose units [1]. This polycationic polymer is nontoxic to humans and has numerous applications, especially in agriculture and the pharmaceutical industry. Its most recent and important application is as nanoparticle for the control of phytopathogenic fungi [2]. Chitosan and its derivatives have a potential as antimicrobial agents to inhibit growth of various plant pathogens [3]. This antimicrobial effect is due to interactions between positively charged chitosan and the negatively charged membrane surface. It has been shown that chitosan binds to negatively charged phospholipids, leading to permeabilization of the plasma membrane [4]. However, the application of chitosan is limited due to its poor water solubility. Efforts to improve the water solubility of chitosan include modification of its grade of acetylation and molecular weight, as well as grafting with more hydrophilic groups [5,6].

Other important biological molecules are oligochitosan (OCH) and glycol chitosan (GCH). Oligochitosan, a short chitosan (MW 5000–8000 Da), is a water soluble derivative that inhibits the growth of some phytopathogenic fungi. It has been used at neutral pH against rot caused by *Alternaria alternata* in *Chinese jujube* [7]. In that study, a concentration of 5 g/L of OCH was considered optimal to inhibit the disease development. In other reports, OCH was fluorescently labeled with 2-aminoacridone and used to demonstrate its internalization in *Phytophthora capsici* [8]. The authors proposed that oligochitosan exerts antifungal activity by penetrating the cell membrane and binding to intracellular DNA and RNA

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molecules. Besides, it was demonstrated that oligochitosan also affected the endomembrane system [9] and caused an increase in NADH dehydrogenase activity in mitochondria, therefore it might be implicated in the mitochondrial dysfunction [10]. On the other hand, glycol chitosan is a chitosan derivative soluble in water over a wide range of pH values and possessing potentially useful biological properties [11]. Its ability to control the decay of apple, lemon, and orange fruits, caused by *Botrytis cinerea* and *Penicillium expansum*, has been reported [12]. Glycol chitosan interacts with the plasma membrane and can be internalized into the cell [13].

Effects of chitosan and its derivatives on the morphology and physiology of the *Ustilaginaceae* family have not been studied previously, although *Ustilaginaceae* species cause economically relevant losses in agriculture. In particular, the dimorphic fungus *Ustilago maydis* causes corn smut disease [14]. In recent years, *U. maydis* has become an excellent model to study different aspects of cell development, and nowadays it is one of the best-characterized phytopathogenic fungi [15]. However, there is only one report on the effect of chitosan on *U. maydis* growth [16]. Therefore, there is little information on the biological activities of polycationic macromolecules against this fungal species. The aim of this study was to evaluate the influence of chitosan, oligochitosan and glycol chitosan on cell development and physiology of *U. maydis*.

2. Materials and methods

2.1. Reagents and solutions

Low molecular weight chitosan (DD ≥ 85%, MW 50–190 kDa), oligochitosan (chitosan oligosaccharide lactate, DD > 90%, average MW 5 kDa) and glycol chitosan (DD ≥ 60%, average MW 250 kDa) were purchased from Sigma–Aldrich (St. Louis, MO). All other chemicals and solvents used were of analytical grade. A stock solution of chitosan (10 mg/mL) was prepared with 1 g of chitosan dissolved in 100 mL of 1% acetic acid and stirred for 24 h. The pH was adjusted to 5.6, and this solution was sterilized by autoclaving [17]. To prepare stock solutions of oligochitosan and glycol chitosan (40 mg/mL), 8 g of each was dissolved in 200 mL of distilled water and stirred for 2 h. pH of both solutions was adjusted to 7.0, and they were sterilized by filtration through 0.45-μm membranes [9]. Aliquots of these stocks were diluted to a final concentration of 1 mg/mL for the experiments.

2.2. Strain and inoculum

U. maydis ATCC 201384 (FB2) was obtained from the American Type Culture Collection and stored at −70 °C in 50% glycerol until use. *U. maydis* was grown at 28 °C on solid YPD medium (1% yeast extract, 0.15% ammonium nitrate, 0.25% bacto peptone, 1% glucose and 2% agar, pH 6.8). A pre-inoculum in liquid YPD medium was obtained by incubating cells from the solid culture for 24 h at 29 °C with stirring at 130 rpm. To obtain an inoculum, cells were washed twice with sterile distilled water, and 50 OD_{600} units/L were inoculated into a minimal medium (MM: 1% glucose, 0.3% potassium nitrate and a salt solution, pH 5.6) or YPD, containing CH, OCH, or GCH. Cells grown in MM or YPD without the antifungal compounds were used as controls.

2.3. Growth kinetics

An aliquot of the pre-inoculum containing 5 OD_{600} units was added to 100 mL of the minimal medium containing one of the antifungals at 1 mg/mL final concentration. The cultures were incubated for 48 h at 29 °C and stirring at 130 rpm. Samples (1 mL) were taken at different times, and cell growth was monitored by measuring the optical density at 600 nm, using MM as a blank. MM without the antifungal compounds was inoculated with the same number of cells and used as a control. Growth curves were also determined with different chitosan concentrations (0.2–100 μg/mL final concentrations).

2.4. Minimum inhibitory concentrations (MICs)

MICs were determined as follow: chitosan and derivatives were diluted with sterile water. Aliquotes of 50 μL of serial dilutions of each compound (from 1 to 5000 μg/mL) were transferred to 96 well plates and mixed with 50 μL of an overnight culture of *U. maydis* in YPD media, to get 5×10^6 cells per well (final volume 100 μL). The plates were incubated for 24 h at 28 °C under stirring. The experiments were carried out by triplicate and MICs were determined using ELISA lector with a 620 nm optical filter [18].

2.5. Scanning electron microscopy (SEM)

U. maydis was cultured for 12 h at 28 °C and 130 rpm in flasks with MM containing each compound at a final concentration of 1 mg/mL. Subsequently, the biomass was recovered by centrifugation at 1540 × *g* for 10 min at 4 °C and washed with a Sörensen phosphate buffer to remove excess medium. Cells were fixed for 2 h at 4 °C with 2% glutaraldehyde dissolved in phosphate buffer. After this, the samples were washed with phosphate buffer containing 5% sucrose and 1% $CaCl_2$, followed by treatment with 1% osmium tetroxide at 4 °C for 1 h and washing twice with phosphate buffer. Once this process was completed, the cells were placed in a critical point dryer and sprayed with CO_2 at 5 °C. After the drying process, the samples were spread on metallic containers and applied on a gold layer under vacuum for 10 min. Then, they were stored in a desiccator at room temperature until use. For the evaluation of morphological changes, a JEOL 5800LV scanning electron microscope was used at 15 kV.

2.6. Transmission electron microscopy (TEM)

Cells were initially treated as described above. After the osmium tetroxide treatment and washing with phosphate buffer, the samples were gradually dehydrated with ethanol using increasing concentrations from 10% to 90% with a 10% step, for 10 min at each concentration, followed by three incubations with absolute ethanol, 20 min each. Cells were placed in mixtures of propylene oxide/Epon resin (2:1, 1:1 and 1:2) for 12 h each. After this, the biomass was embedded into pure Epon resin for 2 h. Polymerization was allowed for 18 h at 60 °C, and then 70-nm sections were made and placed on a copper grid. The sections were successively contrasted in uranyl acetate in ethanol and in a solution of lead for 15 min each, with exhaustive washes between changes. Finally, samples were observed in a transmission electron microscope model JEOL 1010 TEM at 60 kV.

2.7. Oxygen consumption measurement in treated cells

Oxygen consumption by cells in the presence of CH, OCH or GCH was determined polarographically in a 2-mL glass chamber with a Clark-type electrode [19,20]. Experiments were performed in buffer A consisting of 10 mM HEPES-KOH, pH 7.0, 10 mM KH_2PO_4, 20 mM KCl, 5 mM $MgCl_2$ and 1 mM EGTA. The final volume of the reaction mixture was 1.3 mL, and the temperature was maintained at 25 °C using a recirculating water bath. *U. maydis* cells were grown in 100 mL of MM with 1 mg/mL of the antifungal compounds at 29 °C and 130 rpm. Aliquots (10 mL) were taken at 12, 24 and 48 h, and cells were harvested by centrifugation at 3000 × *g* for 10 min. Cells were weighed, diluted to 50% (w/v) with sterile distilled water,