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Pyridine-grafted chitosan derivative as an antifungal agent

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ABSTRACT

Pyridine moieties were introduced into chitosan by nucleophilic substitution to afford *N*-(1-carboxybutyl-4-pyridinium) chitosan chloride (pyridine chitosan). The resulting chitosan derivative was well characterized, and its antifungal activity was examined, based on the inhibition of mycelial growth and spore germination. The results indicated that pyridine chitosan exhibited enhanced antifungal activity by comparison with pristine chitosan. The values of the minimum inhibitory concentration and the minimal fungicidal concentration of pyridine chitosan against *Fulvia fulva* were 0.13 mg/ml and 1 mg/ml, respectively, while the corresponding values against *Botrytis cinerea* were 0.13 mg/ml and 4 mg/ml, respectively. Severe morphological changes of pyridine chitosan-treated *B. cinerea* were observed, indicative that pyridine chitosan could damage and deform the structure of fungal hyphae and subsequently inhibit strain growth. Non-toxicity of pyridine chitosan was demonstrated by an acute toxicity study. These results are beneficial for assessing the potential utilization of this chitosan derivative and for exploring new functional antifungal agents with chitosan in the food industry.

1. Introduction

Chitosan is a natural, inexpensive, and nontoxic polysaccharide with a long-chain backbone comprised of *N*-acetyl glucosamine. It has been utilized in soil amendment and seed treatment, as well as for foliar treatment to control the fungi, *Fusarium oxysporum*, *Fusarium acuminatum*, *Cylindrocladium floridanum*, and *Aspergillus flavus* (Rabea, Badawy, Stevens, Smagghe, & Steurbaut, 2003). The application of chitosan as a bioactive substance to control postharvest fungal disease has also drawn great attention due to public concerns about food safety, overuse of synthesized chemicals, fungicide residue, and emergence of fungicide-tolerant pathogens. However, the remarkably poor solubility of chitosan, caused by the high crystallinity owing to the existence of hydrogen bonds and acetamido groups, limits its wide application. Therefore, it is necessary to modify the structure of pristine chitosan to improve its solubility and other properties.

Extensive efforts have been devoted to the preparation of functional chitosan derivatives by introducing quaternary ammonium moieties onto the chitosan polymer chain, in order to increase the solubility of chitosan in water and increase its antibacterial activities (Rúnarsson et al., 2007). For instance, a water-soluble chitosan derivative, *N*,*N*,*N*-trimethylammonium chitosan chloride (TMChC), has been fabricated and has exhibited effective antibacterial activity against an array of Gram positive and negative bacteria, with moderate cytotoxicity (Mohamed, Sabaa, El-Ghandour, Abdel-Aziz, & Abdel-Gawad, 2013). The antibacterial activity of the chitosan derivatives decreased when the alkyl spacer length between the quaternary ammonium moieties and the chitosan polymer backbone increased (Sahariah et al., 2014). The introduction of quaternary ammonium moieties into *N*-substituted carboxymethyl chitosan (*N*-substituted CMCh) derivatives also enhanced their biological activity, with higher antibacterial activity against Gram-positive bacteria than against Gram-negative bacteria. By comparison with the pristine chitosan, increased antibacterial activities had been acquired upon the use of various quaternary ammonium chitosan derivatives, such as methylated *N*-(4-*N*,*N*-dimethylaminobenzyl) chitosan chloride (MDMBzCh), methylated *N*-(4-pyridylmethyl) chitosan chloride (MPyMeCh) (Sajomsang, Gonil, & Saesoo, 2009), quaternized *N*-(3-pyridylmethyl) chitosan derivatives (Sajomsang, Ruktanonchai, Gonil, & Warin, 2010), *N*-(2-quarternary ammonium)acyl derivatives of chitosan with a quaternary *N*-trialkyl or aromatic ammonium moiety on the 2-position of the *N*-acetyl spacer group (Rúnarsson et al., 2010), quaternized chitosan derivatives with *N*-(3-chloro-2-hydroxypropyl)trimethylam monium chloride (Sajomsang, Tantayanon, Tangpasuthadol, & Daly, 2009), quaternary ammonium chitosan derivatives containing mono or disaccharide moieties (Sajomsang, Gonil, & Tantayanon, 2009), and β-cyclodextrin moiety grafted chitosan (Gonil et al.,

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2011; Sajomsang et al., 2011), chitosan derivatives with quaternary pyridiniumyl against *Botrytis cinerea* (*B. cinerea*) and *Fulvia fulva* (*F. fulva*) with lower minimum inhibitory concentrations (MIC) (Rúnarsson et al., 2010), quaternary *N*-(benzyl) chitosan derivatives with the antimicrobial activity against various important crop-threatening bacteria and fungi (Badawy, Rabea, & Taktak, 2014).

The antifungal properties of chitosan and chitosan derivatives are of interest to the food industry because chitosans are safe biopolymers, suitable for oral administration (Petchsangsai et al., 2011; Zambito, Felice, Fabiano, Di Stefano, & Di Colo, 2013; Zambito et al., 2009). In this study, a new chitosan derivative as an effective antifungal agent with the properties of low toxicity and biodegradability was designed, synthesized and assessed. Pyridine, which is a common heteroaromatic group frequently used as a pharmacophore for agrochemicals (Sajomsang et al., 2010), was introduced into the chitosan backbone, via alkylation, to afford a *N*-alkylpyridinium-based quaternary chitosan salt, *N*-(1-carboxybutyl-4-pyridinium) chitosan chloride (pyridine chitosan). The resulting water-soluble quaternized chitosan was characterized by Fourier transform infrared spectroscopy (FTIR), ultraviolet–visible spectrophotometry (UV–Vis), and nuclear magnetic resonance (NMR) spectroscopy. Its antifungal activities against *B. cinerea* and *F. fulva* were tested *in vitro*, and acute toxicity was assessed.

2. Materials and methods

2.1. Materials

Chitosan with 96.1% degree of deacetylation and a centipoise viscosity of 20 mPa s and chitosan with 95.6% degree of deacetylation and a centipoise viscosity of 423 mPa s (1%, 20 °C) was purchased from Ningbo Zhenhai Haixin Biological Products Co., Ltd. (Zhejiang, China). Other reagents and solvents were purchased from Aladin Reagent Co., Ltd. (Shanghai, China) and were used directly without further purification.

2.2. Synthesis of *N*-(1-carboxybutyl-4-pyridinium) chitosan chloride (**2**)

Two grams of chitosan (423 mPa s) was dissolved in 60 ml of *N*-methylpyrrolidone (NMP) under nitrogen, and 200 μl of 4-chlorobutyryl chloride dissolved in 5 ml of NMP were added dropwise to the solution. The reaction mixture was stirred at room temperature for 3 h. The resulting polymer was precipitated with diethyl ether and washed with methanol and diethyl ether to afford *N*-chlorobutyryl chitosan (**1**). Then, compound **1** was stirred in 200 ml of pyridine under nitrogen atmosphere at 80 °C for 72 h. After the solvent was evaporated, the product was subsequently washed with methanol and diethyl ether, and dried in a freeze-dryer to afford 2.84 g of *N*-(1-carboxybutyl-4-pyridinium) chitosan chloride (**2**).

2.3. Characterization

^{1}H and ^{13}C NMR spectra were recorded at 343 K on a Bruker AVANCE DRX 500 (Bruker Bios pin, Rheinstetten, Germany). The degree of quaternization substitution (DQ) was determined by ^{1}HNMR spectroscopy by following Eq. (1) (Sajomsang, Tantayanon, et al., 2009).

$$\mathrm{DQ}\ (\%) = (\mathrm{Ar}/n)/[\mathrm{H2} + 1/3\mathrm{NHAc}] \times 100 \quad (1)$$

where DQ (%) is the quaternization of *N*-substitution, Ar is the integral area of aromatic protons, *n* is the number of aromatic hydrogen atoms per-substituent, H2 is the integral areas of the protons at the C-2 carbon of GlcN, and NHAc is the integral area of GlcNAc protons.

FTIR spectra were recorded on a Nicolet Nexus 870 spectrometer with KBr pellets. UV–Vis spectra were recorded on a UV–Vis spectrophotometer (UV-2550, Shimadzu, Japan).

2.4. Antifungal activity

The fungi tested in this study, including *B. cinerea* and *F. fulva*, were isolated from spoiled blueberries in our laboratory. According to a previous report (Qiu et al., 2014), the fungi were cultured on potato dextrose agar (PDA) for seven days to achieve sporulation. Then, spores were harvested by pouring sterile water into the slant and stirring with a vortex for 40 s. The suspensions of spores and mycelia were filtered through cotton. The spore concentrations were adjusted to 10^5 colony forming units (CFU)/ml, using a hemocytometer under 400× magnification via inversed fluorescent microscopy (Nikon, Eclipse, TE 2000-S).

Different concentration stock solutions of chitosan derivatives were prepared by dissolving in potato dextrose water (PDW) at pH 6.3. After autoclaving at 121 °C for 15 min, the stock solutions were inoculated with 100 μl of fungal spore suspension (approximately 10^5 CFU/ml) for 5 days in a 28 °C incubator and immediately used in standard MIC assays (Qiu et al., 2014). Samples (100 μl) were removed from all wells of the standard MIC plates and spotted on Petri dishes containing PDA. The plates were incubated for 5 days at 28 °C. The minimal fungicidal concentration (MFC) was defined as the concentration of antifungal agent at which the number of colony forming units was zero (Hawser & Islam, 1999).

The effect of mycelial growth of the fungi was assessed according to the previous literature (Qiu et al., 2014). Mycelial stipitipellis (3 mm in diameter) from 1-week-old fungal cultures were placed in the centers of 90 mm Petri dishes containing 20 ml of PDA with various polymer concentrations, ranging from 0.125 to 64.0 mg/ml, and were then incubated at 28 °C in the dark. Mycelial growth was determined by measuring the colony diameter 7 days after inoculation. Each treatment was replicated thrice, and the experiment was repeated twice. The inhibition percentage of mycelial growth was calculated using Eq. (2) and was expressed as a percentage:

Mycelial growth inhibition (%) was expressed as centrifugal index.

$$\text{Antifungal index}\ (\%) = (1 - D_a/D_b) \times 100\% \quad (2)$$

where D_a is the diameter of the growth zone in the test plates, and D_b is the diameter of the growth zone in the control plate.

2.5. Spore germination

Aliquots of 25 ml of different chitosan solutions containing approximately 10^5 conidia/ml were placed at the centers of multi-well microscope slides, each containing 10 wells. After incubation in the dark at 28 °C for 8 h, the numbers of germinated and non-germinated conidia were recorded with a 400× inversed fluorescent microscope (Nikon, Eclipse, TE 2000-S). The germination percentage was calculated using Eq. (3) and was expressed as a percentage (Qiu et al., 2014):

$$\text{Spore germination inhibition rate}\ (\%) = (N_1 - N_2)/N_1 \times 100\% \quad (3)$$

where N_1 is the observed spore number, and N_2 is spore germination number.

2.6. Scanning electron microscopy (SEM) (Sahariah et al., 2014)

The morphology of pyridine chitosan on *B. cinerea* mycelial growth was investigated, using a regular method for electron microscopy examination. The hyphae (1–2 mm^2) were separately