Synthesis, characterization, and antifungal activity of novel quaternary chitosan derivatives

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ABSTRACT

Three novel quaternary chitosan derivatives were successfully synthesized by reaction of chloracetyl chitosan (CACS) with pyridine (PACS), 4-(5-chloro-2-hydroxybenzylideneamino)-pyridine (CHPACS), and 4-(5-bromo-2-hydroxybenzylideneamino)-pyridine (BHPACS). The chemical structure of the prepared chitosan derivatives was confirmed by Fourier transform infrared (FT-IR) and 13C nuclear magnetic resonance (13C NMR) and their antifungal activity against Cladosporium cucumerinum, Monilinia fructicola, Colletotrichum lagenarium, and Fusarium oxysporum was assessed. Comparing with the antifungal activity of chitosan, CACS, and PACS, CHPACS and BHPACS exhibited obviously better inhibitory effects, which should be related to the synergistic reaction of chitosan itself with the grafted 2-[4-(5-chloro-2-hydroxybenzylideneamino)-pyridyl]acetyl and 2-[4-(5-bromo-2-hydroxybenzylideneamino)-pyridyl]acetyl.

1. Introduction

Chitosan has attracted people's attention for its physiochemical characteristics and its bioactivities.1-6 The antimicrobial properties of chitosan and its derivatives have been widely explored.7-9 To improve the antimicrobial activity of chitosan, researchers have prepared many derivatives which showed higher antimicrobial activities than chitosan, but the antimicrobial activities of these materials are still lower than those of the antimicrobial agents currently used.10-15

N,O-Acyl chitosan derivatives were synthesized and their fungal activities against plant fungus were examined. The derivatives were more active against the tested plant pathogens than chitosan itself.16 In addition, many reports have demonstrated that aromatic nucleus Schiff bases have good bioactivities.17,18 It is also reported that Salicylaldehyde derivatives, with one or more halo-atoms in the aromatic ring, showed a variety of biological activities.19 We describe here the preparation of three novel derivatives of chitosan including the above active groups, (2-pyridyl)acetyl chitosan chloride (PACS), 2-[4-(5-chloro-2-hydroxybenzylideneamino)-pyridyl]acetyl chitosan chloride (CHPACS), and 2-[4-(5-bromo-2-hydroxybenzylideneamino)-pyridyl]acetyl chitosan chloride (BHPACS) (Scheme 1), as well as their antifungal activity against four plant-threatening pathogenic fungi Cladosporium cucumerinum, Monilinia fructicola, Colletotrichum lagenarium, and Fusarium oxysporum that employed the method of Guo.14 Besides, the application of chitosan is limited for its poor solubility in water or high pH region. Therefore, taking low molecular weight chitosan with high solubility and low viscosity in water at physiologically acceptable pH values as starting material can enlarge the scope of application. In this paper, we used water-soluble low molecular chitosan as starting material. The derivatives were expected to have better solubility and antifungal activity.

2. Materials and methods

2.1. Materials

Chitosan was purchased from Qingdao Baicheng biochemical Corp. (China). Its degree of deacetylation was 97% and the viscosity-average molecular weight was 2.0 × 10^5. The water-soluble chitosan was prepared in our laboratory by the method of hydrogen peroxide (H₂O₂) hydrolysis and the viscosity-average molecular weight was 7.0 × 10^3. The other reagents were of analytical grade and used without further purification.

2.2. Analytical methods

The Fourier transform infrared (FT-IR) spectrum was measured in KBr pellets with a JASCO FT-IR-4100 instrument. The 13C nuclear magnetic resonance (13C NMR) spectrum was measured with a
Bruker AVIII-500 spectrometer. PACS had well water solubility, but CHPACS and BHPACS had slight water solubility. So the $^{13}$C NMR was carried out in D$_2$O (PACS) and in DMSO (CHPACS and BHPACS).

### 2.3. The synthesis of the derivatives of chitosan

The synthesis of 5-chlorosalicylaldehyde and 5-bromosalicylaldehyde was, respectively, carried out according to the procedure of Liu et al. and Liang and Chin.20 The chloracetyl chitosan (CACS) was synthesized referring to the procedure of Hu et al.22 1.61 g chitosan was dispersed in 100 mL N-methyl-2-pyrrolidone (NMP) and stirred for 12 h at room temperature (rt). Then 0.02 mol chloracetyl chloride was added. After stirring for 24 h at rt, the solution was precipitated in ether and the precipitate was washed with methanol and ether by turns and lyophilized. The synthesis of PACS: a solution of 0.3 g CACS and 0.5 mL pyridine in 20 mL dimethyl sulphoxide (DMSO) was stirred for 24 h at 60 °C. The solution was collected by precipitation with excess acetone and the product was lyophilized. CHPACS and BHPACS were synthesized as follows: a solution of 0.01 mol 5-chlorosalicylaldehyde or 5-bromosalicylaldehyde and equimolar amount of 4-amino-pyridine were refluxed in 30 mL ethanol for 4 h. Then the solvent was evaporated under reduced pressure and the product was dissolved by adding 20 mL DMSO. After the product dissolved under stirring, 0.3 g CACS was added. The solution was stirred at 60 °C for 24 h and then precipitated with excess acetone and the precipitate was filtered. The unreacted aldehyde, amine, and other outgrowth were extracted in a Soxhlet apparatus with ethanol for two days. The product was lyophilized.

### 2.4. Antifungal assays

Antifungal assays were performed based on the method of Guo et al.14 Briefly, the compounds were dispersed in distilled water at a concentration of 5 mg/mL. Then, each samples (chitosan, CACS, PACS, CHPACS, and BHPACS) solution was added to sterilized potato dextrose agar to give a final concentration of 100, 500, and 1000 μg/mL.1 After the mixture was cooled, the mycelium of fungi was transferred to the test plate and incubated at 27 °C for 2–3 days. When the mycelium of fungi reached the edges of the control plate (without the presence of samples), the antifungal index was calculated as follows:

\[
\text{Antifungal index} \% = \left(1 - \frac{D_a}{D_b}\right) \times 100,
\]

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. Each experiment was performed three times, and the data were averaged. The Scheffe method was used to evaluate the differences in antifungal index in antifungal tests. Results with $P<0.05$ were considered statistically significant.23

### 3. Results and discussion

#### 3.1. Structure of the chitosan derivatives

Figure 1 presents the comparison of the transmission FT-IR spectra for CACS, PACS, CHPACS, and BHPACS with the original chitosan.
As far as the FT-IR spectra of CACS and chitosan are concerned, the characteristic absorbance of –NH₂ at 1600 cm⁻¹ disappeared. The stretching vibration of the carbonyl groups C=O of acylamide at 1666 cm⁻¹ and of the ester group at 1750 cm⁻¹ could be obviously observed.²⁴²⁵ New peaks at 787 cm⁻¹ were assigned to the C–Cl group. It indicated that CACS was obtained by the acylated reaction between chloracetyl chloride and the groups (–NH₂, –OH) of chitosan. After pyridine grafted onto the CACS, the characteristic absorbance of C–Cl at 787 cm⁻¹ disappeared and new peaks at 1496, 779, and 3081 cm⁻¹ were due to the characteristic absorbance of pyridine²⁶ appeared in the spectra of PACS. In the spectrum of CHPACS, new peaks appeared at 1481 cm⁻¹, 3135 cm⁻¹ which were attributed to aromatic ring stretching vibrations and C–H stretching vibrations, respectively. The out-of-plane pyridine deformation vibrations were found at 767 cm⁻¹ and the peak at 829 cm⁻¹ was due to benzene bending. Another prominent peak at 1284 cm⁻¹ was assigned to the C–O of phenolic hydroxyl group.²⁶²⁷ The characteristic absorbance of C–Cl at 787 cm⁻¹ disappeared and the peaks of carbonyl groups of acylamide and ester were bathochromic shifted to 1658 and 1712 cm⁻¹, respectively, due to the graft of the 4-(5-chloro-2-hydroxybenzylideneamino)-pyridine.²⁸ For the similar reason, the C–Cl characteristic absorbance at 787 cm⁻¹ disappeared in the FT-IR spectra of BHPACS. And both the appearing of the new peaks at 1477 cm⁻¹, 1284 cm⁻¹, 829 cm⁻¹, 767 cm⁻¹ and the carbonyl groups of acylamide and ester bathochromic shifting to 1658 and 1712 cm⁻¹ indicated the successful synthesis of BHPACS. The thirteen C NMR spectrum of PACS, CHPACS, and BHPACS (Fig. 2) further confirmed the success of the preparation.

Spectral data for PACS. ¹³C NMR/H₂O: δ 173.1 ppm (C=O of ester group), 166.0 ppm (C=O of acylamide).²⁵ δ 146.8, 145.5 and 127.8 ppm (pyridine ring carbons), ²⁸ δ 55.8–99.7 ppm (pyranose rings), δ 60.1, 38.3 ppm (methylene carbon of –OCOCH₂– and –NH₂COCH₂–).³⁰

Spectral data for CHPACS. ¹³C NMR/DMSO: δ 166.1, 166.6 ppm (C=O of ester group and acylamide),²⁵ δ 159.3, 144.5, 109.3 ppm (pyridine ring carbons), δ 159.3 ppm (carbon of N=CH–), δ 118.9, 120.4, 122.6, 131.0, 132.6, 159.3 ppm (benzene ring carbons).³¹ δ 58.6–101.1 ppm (pyranose rings). The peak of methylene carbon in –OCOCH₂– was included in the peaks at about 60 ppm and that of methylene carbon in –NH₂COCH₂– was covered by peaks of DMSO.³⁰

Spectral data for BHPACS. ¹³C NMR/DMSO: δ 166.0, 166.5 ppm (C=O of ester group and acylamide),²⁵ δ 159.4, 144.5, 109.6 ppm (pyridine ring carbons), δ 159.4 ppm (carbon of N=CH–), δ 119.1–135.3, and 159.4 ppm (benzene ring carbons).³¹ δ 58.8–100.9 ppm (pyranose rings). The chemical shift of methylene carbon in –OCOCH₂– was included in the peaks at about 60 ppm and that of methylene carbon in –NH₂COCH₂– was covered by peaks of DMSO.³⁰

### 3.2. Solubility of the chitosan derivatives

The water solubility of chitosan and the derivatives is listed in Table 1. Because of the decrease of hydrophilic hydroxyl and amino groups, CACS exhibited soft water solubility. After pyridine substituted the chlorine in CACS, PACS was obtained and quaternary ammonium salt structure was formed. Therefore, PACS has favorable water solubility. The solubility of CHPACS and BHPACS was less than that of material chitosan, maybe due to the hydrophobic moiety at the end of the molecular chains.
3.3. Antifungal activity

The most economically important seed or plant diseases are caused by the action of fungi. The need to find eco-friendly treatments to avoid the use of aggressive chemicals that cause contamination is growing. Here, we tested the antifungal activities of the starting chitosan, CACS, PACS, CHPACS, and BHPACS against four kinds of plant fungus. The results are demonstrated as follows.

3.3.1. Antifungal activities of chitosan, CACS, PACS, CHPACS, and BHPACS against *C. cucumerinum*

As shown in Figure 3, chitosan and its derivatives had antifungal activities at all the tested concentrations against *C. cucumerinum*. CACS showed better antifungal activity than chitosan at 1000 µg/mL with the inhibitory index 41.4%. The inhibitory indices of PACS, CHPACS, and BHPACS enhanced with increasing concentration. With the inhibitory indices 100% at 500 µg/mL, CHPACS and BHPACS exhibited much better antifungal activities than chitosan and CACS. However, the antifungal activity of PACS against *C. cucumerinum* did not reveal apparent improvement than chitosan and CACS.

3.3.2. Antifungal activities of chitosan, CACS, PACS, CHPACS, and BHPACS against *M. fructicola*

As shown in Figure 4, chitosan, CACS, and PACS had soft antifungal activities against *M. fructicola* just as *C. cucumerinum* was concerned. The inhibitory indices of chitosan, CACS, PACS, CHPACS, and BHPACS at 500 µg/mL were 6.9%, 14%, 19.1%, 100%, and 100%, respectively. The inhibitory indices of chitosan, CACS, PACS, CHPACS, and BHPACS at 1000 µg/mL were 9.3%, 14%, 21.5%, 100%, and 100%, respectively. It indicated that CHPACS and BHPACS have potent activities. Comparing the difference of the compounds structure, we can speculate that the increased antifungal activities of CHPACS and BHPACS may benefit from 5-chloro-2-hydroxylbenzylideneamino and 5-bromo-2-hydroxybenzylideneamino groups in the compounds.

3.3.3. Antifungal activities of chitosan, CACS, PACS, CHPACS, and BHPACS against *C. lagenarium*

Figure 5 shows the antifungal activity against *C. lagenarium* of chitosan, CACS, and all the derivatives. All the samples have antifungal activity against *C. lagenarium*. And the inhibitory indices of all the samples mounted up with increasing concentration. The inhibitory indices of chitosan, CACS, PACS, CHPACS, and BHPACS at 500 µg/mL were 32.0%, 26.6%, 15.3%, 45.3%, and 63.3%, respectively, and 36.6%, 36.6%, 30.2%, 71.0%, and 75.8%, respectively, at 1000 µg/mL. It indicated that the antifungal activities of CHPACS and BHPACS against *C. lagenarium* were markedly better than those of chitosan, CACS, and PACS. The results, again, suggested that 5-chloro-2-hydroxybenzylideneamino and 5-bromo-2-hydroxybenzylideneamino groups maybe antifungal function groups.

3.3.4. Antifungal activities of chitosan, CACS, PACS, CHPACS, and BHPACS against *F. oxysporum*

*F. oxysporum*, also referred to as Panama disease or Agent Green, is a fungus that causes *Fusarium* wilt disease in more than a hundred species of plants. It does so by colonizing the water-conducting vessels (xylem) of the plant. As a result of this blockage and breakdown of xylem, symptoms such as leaf wilting, yellowing, and eventually plant death appear in plants. Therefore, the study on antifungal agents is significative. The fungicidal activities of the compounds toward *F. oxysporum* are depicted in Figure 6. As shown in Figure 6, the diversification trend of the

| Table 1 Water solubility of CACS, PACS, CHPACS, and BHPACS |
|----------------|----------------|----------------|
| Compounds    | Water solubility (%) | Test time (min) |
| Chitosan     | 30.5            | 5              |
| CACS         | 1.5             | 20             |
| PACS         | 25.5            | 5              |
| CHPACS       | 6.5             | 10             |
| BHPACS       | 6.0             | 10             |

Figure 3. The antifungal activities of chitosan, CACS, PACS, CHPACS, and BHPACS against *C. cucumerinum*.

Figure 4. The antifungal activities of chitosan, CACS, PACS, CHPACS, and BHPACS against *M. fructicola*.

Figure 5. The antifungal activities of chitosan, CACS, PACS, CHPACS, and BHPACS against *C. lagenarium*.
antifungal activities against *F. oxysporum* of chitosan and its derivatives resembled that against *C. lagenarium*. The inhibitory indices of CHPACS and BHPACS were, respectively, 57.0%, 58.9% at 500 μg/mL and 77.2%, 72.2% at 1000 μg/mL. The inhibitory indices of chitosan and CACS were only 22.8%, 17.0% at 500 μg/mL and 44.3%, 35.6% at 1000 μg/mL, respectively.

Above results indicated that the antifungal activity of CACS and PACS against the four investigated fungi did not reveal apparent improvement than that of chitosan. The reason was maybe, for CACS, the graft of chloracetyl onto the chitosan led to the contents improvement than that of chitosan. The reason was maybe, for PACS against the four investigated fungi did not reveal apparent indices of chitosan and CACS were only 22.8%, 17.0% at 500 μg/mL and 44.3%, 35.6% at 1000 μg/mL, respectively.

In this work, three novel quaternized chitosan derivatives were synthesized successfully, in which, CHPACS and BHPACS were two effective antifungal derivatives of chitosan and the inhibitory indices against *C. cucumerinum*, *M. fruticola*, *C. lagenarium*, and *F. oxysporum* ranged from 45.3% to 100% at 500 μg/mL and 71.0% to 100% at 1000 μg/mL. The inhibitory indices of the two compounds enhanced with increase in concentration, and the inhibitory index 100% was observed at 500 μg/mL against *C. cucumerinum* and *M. fruticola*. Moreover, the antifungal activities of CHAPCS and BHPACS were noticeably better than those of chitosan, CACS, and PACS which may be due to the presence of 5-chloro-2-hydroxybenzylideneamino and 5-bromo-2-hydroxybenzylideneamino groups. It was in accordance with the conclusions of Guo et al.34