Antifungal Properties of Chitosan Salts in Laboratory Media

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Received 3 December 2009; accepted 14 October 2010
DOI 10.1002/app.33612
Published online 2 November 2011 in Wiley Online Library (wileyonlinelibrary.com).

ABSTRACT: Some aliphatic carboxylic acids were used to produce chitosan (CS) salts by reaction with CS, and their antifungal activity against three kinds of phytopathogens was estimated by hypha measurement *in vitro*. The fungicidal assessment showed that all of the CS salts had excellent activity against the tested fungi. Their inhibitory indices were 41.15–64.15, 56.25–76.56, and 35.94–68.75% for *Cladosporium cucumerinum* (Ell.) et Arthur, *Monilinia fructicola* (Wint.) Honey, and *Fusarium oxysporum* sp. Cucumis sativus L., respectively, at 1000 μg/mL; these indices were higher than that of CS. It was confirmed that the amino groups' protonation was important for the antifungal activity of CS derivatives. The substituted groups with stronger electronegativity drew more electrons from the nitrogen atoms in the derivative molecules, which relatively strengthened the polycationic character of the CS derivatives. Therefore, the antifungal activity of the CS salts was improved. © 2011 Wiley Periodicals, Inc. J Appl Polym Sci 124: 2501–2507, 2012

Key words: biological applications of polymers; biopolymers; FT-IR; NMR

INTRODUCTION

Chitin, an α,β-(1,4)-d-linked polymer of *N*-acetylglucosamine, is one of the most abundant natural resources found on the earth after cellulose. Chitosan (CS) is a deacetylated derivative of chitin. CS is actually a copolymer of 2-acetamido-2-deoxyglucopyranose units linked by β-(1-4) linkages and 2-amino-2-deoxyglucopyranose units.[1] As a natural and important renewable resource that is both nontoxic and biodegradable, CS has attracted much research attention in the last 30 years. Among the various bioactivities of CS, the antimicrobial activity of CS and its derivatives against different groups of fungi has received considerable attention in recent years. It has been shown to be useful in many different areas as an antimicrobial compound in agriculture, as an additive in the food industry, and more recently, as a pharmaceutical agent in biomedicine.[2]

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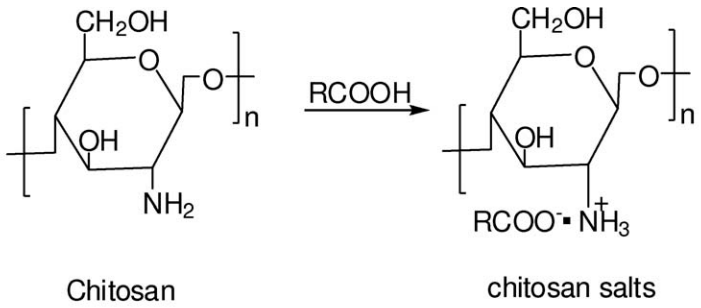
Contract grant sponsor: Knowledge Innovation Program of the Chinese Academy of Sciences; contract grant number: kzcx2-yw-225.
Contract grant sponsor: Special Prize of President Scholarship for Postgraduate Students of Chinese Academy of Sciences.

At a concentration of 2.5 mg/mL, CS completely inhibited the growth of *Monosporascus canonballus* and *Pythium irregulare*.[3] CS markedly reduced the decay of strawberries caused by *Botrytis cinerea* or *Rhizopus stolonifer*.[4] Although these studies proved that CS had antifungal activities, it presents antibacterial activities only in acidic media because of its poor solubility above pH 6.5, especially for high-molecular-weight CS. Indeed, the antifungal effect of CS against *Saccharomyces cerevisiae* under neutral conditions was lower than that under acidic conditions.[5] It cannot be ignored that some acid also have antibacterial activities, which can strengthen the antifungal activities of CS in investigative experiments. Thus, water-soluble CS derivatives, which are soluble in both acidic and basic physiologic circumstances, have become the focus of studies on CS applications. They might be good candidates for polycationic biocides. Some CS salts produced from CS and organic acids can inhibit the growth of some microorganisms. CS–glutamate reduced the growth rate of *Mucor racemosus* and prevented the growth of three strains of *Byssochlamys*.[6] CS–lactate inhibited the growth of *Saccharomyces bayanus* and *Saccharomyces unisporus* at concentrations of 5 and 1 g/L, respectively.[7] Therefore, these CS salts are worthy of further study as natural preservatives for foods or biocides for agriculture.

The previous data confirmed that the alkyl chain length strongly affects the antimicrobial activity of



Chitosan chitosan salts

CS–formate: R=H; CS–acetate: R=CH_3–; CS–propionate: R=CH_3CH_2–; CS–butyrate: R=$CH_3(CH_2)_2$–; CS–valerate: R=$CH_3(CH_2)_3$–

Scheme 1 Synthesis of the CS salts.

CS derivatives, where *N*-propyl-*N*,*N*-dimethyl CS was stronger than *N*,*N*,*N*-trimethyl CS against *Escherichia coli*.[8] Moreover, according to the literature, the increased activity should be attributed to the positive charge density of quaternized CS strengthened after it was quaternized.[9] It seemed that the alkyl chain can strengthen the cation intensity in CS derivatives. To further investigate the relationship between the antifungal activity and the charge density of the cation in CS derivatives, in this study, some aliphatic carboxylic acids, including formic acid, acetic acid, propanoic acid, butyric acid, and valeric acid, were used to produce CS salts. Their antifungal activities against three kinds of phytopathogens were estimated by hypha measurement *in vitro*. *Cladosporium cucumerinum* (Ell.) et Arthur and *Monilinia fructicola* (Wint.) Honey were the most sensitive fungi to CS and its salts. The filamentous fungus, *Fusarium oxysporum* sp. Cucumis sativus L., was the most resistant to the antifungal effects of CS and its salts. Furthermore, it seemed that the aliphatic chain could influence the antifungal activities of the CS salts because of the strengthened cation intensity in the derivatives.

EXPERIMENTAL

Materials and methods

CS was purchased from Qingdao Baicheng Biochemical Corp. (China). Its degree of deacetylation was 97%, and the viscosity-average molecular weight was 7.0×10^4. Formic acid, acetic acid, propanoic acid, butyric acid, and valeric acid were purchased from Sigma-Aldrich Chemical Co. The other reagents were all analytical grade and were used without further purification. The IR spectra were measured on a Jasco-4100 Fourier transform infrared (FTIR) spectrometer (Tokyo, Japan, provided by JASCO China (Shanghai), Co., Ltd. Shanghai, China) with KBr disks, and ^{1}H-NMR spectroscopy was run on a Bruker AVIII-500 spectrometer (Fällanden, Switzerland, provided by Bruker BioSpin CN / Bruker (Beijing) Tech. and Serv. Co., Ltd. Beijing, China.). The electronegativity of various aliphatic groups in CS salts refers to ref. 10. The average viscometric molecular weight of CS was estimated from the intrinsic viscosity determined in the solvent, 0.1*M* CH_3COOH/0.2*M* NaCl, with the Mark–Houwink parameter ($\alpha = 0.96$, $K\eta = 1.424$ at 25°C), where the intrinsic viscosity is expressed in milliliters per gram.[11] "Kη" a parameter of Marke-Houwink equation which could give the average molecular weight "M" of chitosan when the intrinsic viscosity of chitosan solvent was known.

Preparation of the CS salts

The CS salts were synthesized as follows:[12] CS (20 mmol) was ground in 20 mmol of aliphatic carboxylic acid with water for 30 min Cottonlike products were precipitated with excess acetone, and the CS salts were obtained by drying at 60°C *in vacuo* for 12 h (Scheme 1).

Microorganisms

A total of three pathogenic fungi associated with plant diseases were assembled for this study. The phytopathogenic fungi *C. cucumerinum* (Ell.) et Arthur, *M. fructicola* (Wint.) Honey, and *F. oxysporum* sp. Cucumis sativus L. were kindly gifted from Qingdao Academy of Agricultural Sciences.

Antifungal essays

The fungistatic activity of CS and its salts was determined by a radial hyphal growth bioassay.[13] First, samples of CS were dissolved in dilute hydrochloric acid, and their salts were in distilled water at a concentration of 5 mg/mL. Then, the samples were added to sterile Petri dishes (diameter = 9 cm) containing sterilized potato dextrose agar to give final concentrations of 50, 500, and 1000 μg/mL. The

plates were inoculated with plugs with a diameter of 5 mm; the plugs were taken from the margins of 3- or 5-day-old colonies of various fungi on potato dextrose agar. Three replicates for each sample concentration were tested. The control plates were also inoculated with the fungi. When it came to the control plates, an identical volume of dilute hydrochloric acid replaced the samples of CS, and the same volume of distilled water replaced the CS salts. All of the plates were incubated in the dark at 27°C. The radial colony growth was measured when the mycelium of fungi reached the edges of the control plate. The antifungal index was calculated as follows:

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

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.

Water solubility measures

Water solubility measures were performed on the basis of the methods of Letinski et al.[14] Briefly, bottles with rubber stoppers were rinsed appropriately and were air-dried in a laboratory fume hood. Before the addition of the respective test CS salts, these bottles, obturated by stoppers, were filled with opportune volumes of test water overnight at 25°C to lead the water to equilibrate to the test temperature. Under the protection of nitrogen, water was stirred with no visible vortex, and the individual compound was added slowly until there was visual evidence of particles suspended in the water column. After the solution was further stirred for 15 min, the samples were centrifuged, and sufficient saturated solutions of the CS salts were obtained. We took 10 mL of the saturated solution to freeze out for analytical determination. Corresponding control vessels were prepared with no addition of the test material. Each experiment was conducted three times.

Statistical analysis

All data are expressed as means plus or minus the standard deviation. The data were analyzed by an analysis of variance ($p < 0.05$), and the means were separated by Student–Newman–Keuls. The results were processed by the computer programs Excel (Microsoft Co., Ltd. Beijing, China) and Statistica software (SPSS) (IBM Co., Ltd. Beijing, China).

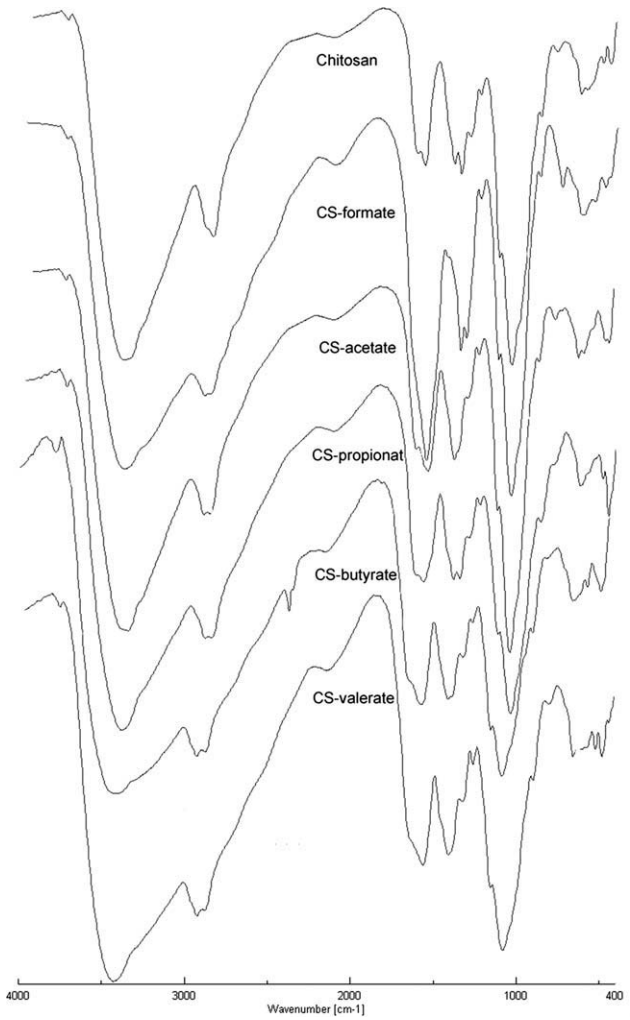


Figure 1 FTIR spectra of the CS and CS salts.

RESULTS AND DISCUSSION

Characterization of the CS salts

In the FTIR spectra of CS and the salts (Fig. 1), CS was typically characterized by absorption regions as follows:[15] the major peaks of CS at about 896, 1087, and 1600 cm^{-1}, belonging to pyranose rings, glucoside, and amine groups, respectively, were identifiable. The peak at about 3400 cm^{-1} became wide and weak for the CS salts because of the formation of $-NH_3^+$. Moreover, the characteristic peak of the amine (N—H) vibration deformation, which appeared at about 1600 cm^{-1} for CS, disappeared for the salts. Instead, two new peaks at 1589–1562 and 1415–1407 cm^{-1} appeared; these were attributed to the stretching vibration for $-NH_3^+$ (1589–1562 cm^{-1}) and $-COO^-$.[16] Furthermore, the large peak at 1589 cm^{-1} for CS–butyrate might have shown that the generation of carboxylate between $-NH_3^+$ and $-COO^-$ groups.[17] The structure of the synthesized products was further demonstrated by ^{1}H-NMR spectroscopy

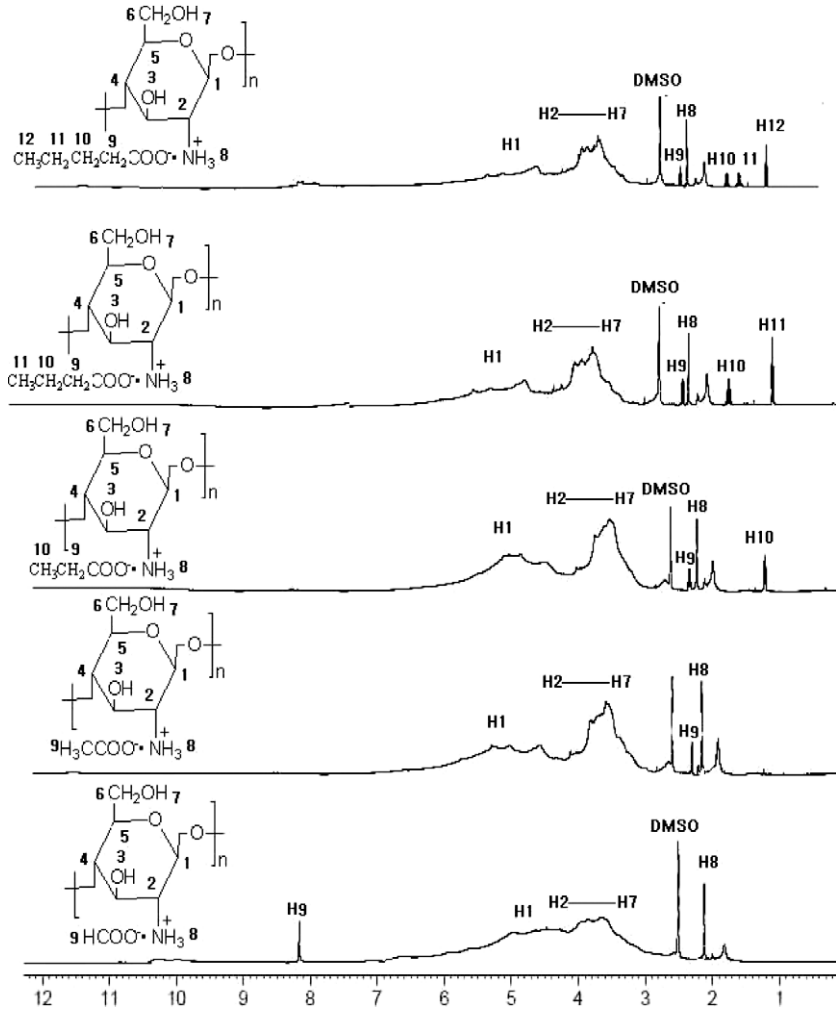


Figure 2 ^{1}H-NMR spectra of CS salts. (DMSO = dimethyl sulfoxide).

(Fig. 2). As marked in Figure 2, the chemical shifts of the aliphatic chains of various CS salts and quaternized amides appeared. The signals at 1.8 ppm (ppm is the unit of chemical shift of NMR results) indicated the N—H groups, which did not react, and this also revealed the degree of substitution of the CS salts.[18] The aforementioned results demonstrated that the quaternized CS derivatives were obtained.

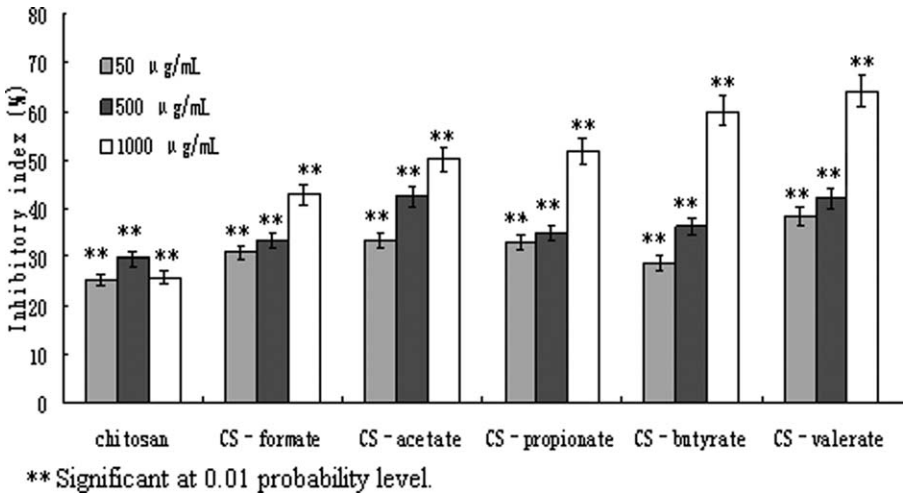


Figure 3 Antifungal activity of the CS and CS salts against *C. cucumerinum* Ell. et Arthur.

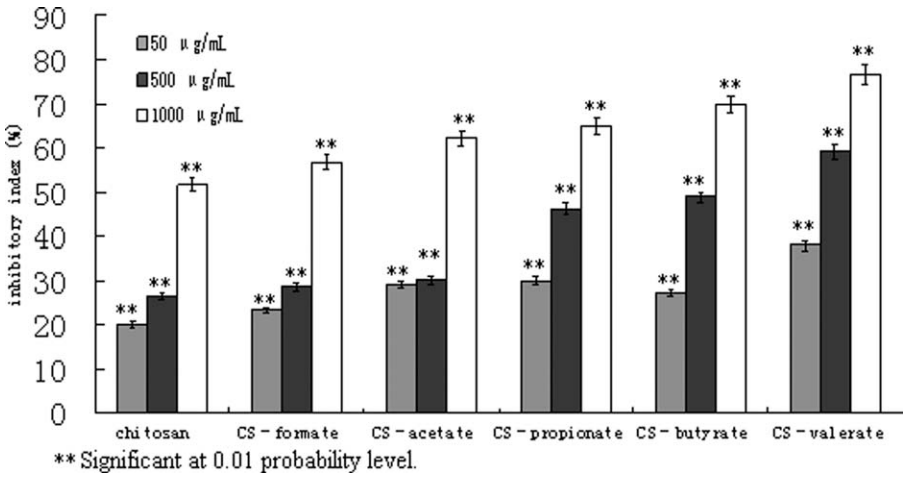


Figure 4 Antifungal activity of the CS and CS salts against *M. fructicola* (Wint.) Honey.

Antifungal activities against various plant pathogenic fungi

Figures 3–5 demonstrate the antifungal activity of CS and its salts against three different plant pathogenic fungi. All of the samples exhibited concentration-dependent inhibitory effects on the hyphal growth of the phytopathogenic fungi.

As shown in Figures 3 and 4, *C. cucumerinum* (Ell.) et Arthur and *M. fructicola* (Wint.) Honey were the most sensitive fungi to CS and its derivatives. The compounds inhibited the growth of the fungi at low concentrations (50 μg/mL). The inhibitory indices for CS–valerate against these two fungi were 64.15 and 76.56%, respectively, at 1000 μg/mL. An earlier article reported that the spraying of aqueous chemical agent solutions on the upper surface of the leaves of cucumber plants induced systemic resistance to *C. cucumerinum.* However, the chemical solution tested caused restricted necrotic lesions on the leaves.[19] Recently, some compounds extracted from several medicinal plants were investigated for their antifungal activity against *C. cucumerinum*. However, these compounds' activity were not quantitative in the literature.[20–22] The data obtained indicated that all kinds of CS salts in this study could effectively inhibit the growth of *C. cucumerinum.*

Similarly, as shown in Figure 4, the CS salts showed inhibitory effects on the growth of *M. fructicola,* which seemed to be more sensitive to the compounds. Brown rot caused by *M. fructicola* is a major disease on stone fruit, including apricots, cherries, and peaches. In recent years, substantial efforts have been made to find effective biocontrol agents from microorganisms to control the brown rot of stone fruit.[23] To this day, there are only a few microorganisms that are effective for controlling the growth of *M. fructicola*.[24] Their acceptance by consumers is still debatable. As shown in this article, the CS salts showed a 60–80% inhibitory effectiveness on the growth of *M. fructicola* at 1000 μg/mL.

Compared with the *C. cucumerinum* (Ell.) et Arthur and *M. fructicola, F. oxysporum* was less sensitive to CS and the synthesized derivatives. Previous studies have found that with preharvest and postharvest treatments, CS significantly reduced the disease incidence caused by *F. oxysporum* f. sp. radicis-

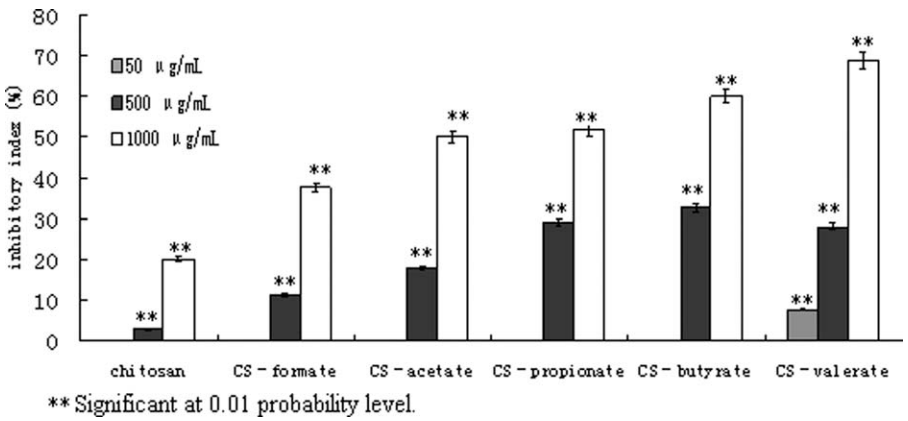


Figure 5 Antifungal activity of the CS and CS salts against *F. oxysporum* Cucumis sativus L.

TABLE I
Electronegativity of Different R Groups in the CS Salts

R group	H	$-CH_3$	$-CH_2CH_3$	$-(CH_2)_2CH_3$	$-(CH_2)_3CH_3$
Electronegative activity[a]	2.1	2.239	2.271	2.278	2.280

[a] Taken from ref. 10.

lycopersici,[25–27] although similar results were not found in *in vitro* experiments in this study. As shown in Figure 5, CS showed almost no inhibitory effect on the growth of the fungus at the tested concentration. The CS salts exhibited a higher antifungal activity at 500 and 1000 μg/mL, especially CS–valerate, whose inhibitory index was 68.75% at 1000 μg/mL.

On the basis of the results from our experiments and those from the literature, we believe that the antifungal activity of the CS salts was stronger than that of CS. Previous reports also revealed that CS salts could reduce the growth rate of some fungi.[6,7] Furthermore, according to the literature, the alkyl chain length strongly affected the antimicrobial activity of the CS derivatives.[8] Herein, we noticed that the increasing alkyl chain length in the derivatives led to a significant increase in the activity against the fungi tested in this study.

Cation and solubility of CS salts influence on the antifungal activity against plant pathogenic fungi

Recently, the antifungal activity of chitosan and its derivatives has attracted a lot of attention. However, the mechanism for the antifungal activity is still unclear. CS is unique for its polycationic characteristics, with free $-NH_2$ groups, which can be converted into $-NH_3^+$ forms in acid media. Most investigations have been done in acidic media to evaluate the antifungal activities. The protonation of amino groups to form $-NH_3^+$ is very crucial. Indeed, the antifungal effect of CS against *S. cerevisiae* under neutral conditions was lower than that under acidic conditions.[5] The cationic amino groups may interact with the anionic components of the cell wall, such as glucan, mannan, proteins, and lipids; this leads to the formation of an impervious layer around the cell. The layer could prevent the transport of essential nutrients, such as glucose, into the cell and may also disturb the cell wall and, thereby, cause severe leakage of the cell constituents and, ultimately, cell death.[28]

Previous studies have proven that quaternized CS had a higher cationic charge than CS, which could enhance the antifungal activity.[9] In this study, the antifungal activities of the CS salts were more pronounced than that of CS. Different antifungal activities of the derivatives were attributed to their different structures. As Scheme 1 shows, the nitrogen atoms in the CS salts had different positive charges because of different R groups in their molecules. Different R groups had their own electronegativity (Table I). Group electronegativity is the electrophilic characteristic of a group, and it is a very significant parameter for studying the structure and properties of organic compounds. As Table I shows, the electronegativity of different R groups in the quaternized chitosans (QCS) was found to be in the order of $-(CH_2)_3CH_3 > -(CH_2)_2CH_3 > -CH_2CH_3 > -CH_3 >$ H. Groups substituted with stronger electronegativity would draw more electrons. The CS salts may have had different cationic charges because of the substituted groups with different electronegativities. The derivatives were more prone to interaction with anionic components of the cell wall of fungi. As a result, they would have had stronger antifungal activities. Meanwhile, the enhanced antifungal activities might have had something to do with the CS salts' better solubility (Table II), as the solubility of such compounds play an important factor in their biological activity. As revealed in Table II, compared with CS, being unsolvable, the organic salts all exhibited some water solubility, which ranged from 21.7 to 23.2%; this may have contributed to the strengthened antifungal activity. However, this influence on the antifungal index between the CS salts may have been less than minor because the discrepancy of different water solubilities among these compounds was no more than 2%.

CONCLUSIONS

In summary, in this study, CS salts were produced by the reaction of CS with aliphatic carboxylic acids,

TABLE II
Water Solubility of the CS Salts

Compound	Water solubility (%)
CS–formate	23.2
CS–acetate	22.7
CS–propionate	21.3
CS–butyrate	22.0
CS–valerate	21.7

Test time = 15 min; test temperature = 25°C.

and their antifungal activities against three kinds of phytopathogens were estimated by hypha measurement *in vitro,* respectively. The data showed that the derivatives with stronger electronegativity groups had stronger antifungal activities. We concluded that the antifungal activity was influenced by the positive charges strengthened by the electronegativity of the substituted groups in the CS salts. The data obtained in the *in vitro* models clearly established the antifungal potency of all kinds of CS salts and indicated that the most recommended derivative should be CS–valerate because it exhibited the best inhibitory index against the pathogenic fungi. However, comprehensive studies need to be carried out to ascertain the safety of CS salts in experimental animal models and field experiments.

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