Evaluation of quaternary ammonium chitosan derivatives differing in the length of alkyl side-chain: Synthesis and antifungal activity

Lijie Wei a,b, Yingqi Mi a,b, Jingjing Zhang a,b, Qing Li a, Fang Dong a,⁎, Zhongyang Guo a,b,⁎⁎

a Key Laboratory of Coastal Biology and Bioresource Utilization, Yantai Institute of Coastal Zone Research, Chinese Academy of Sciences, Yantai 264003, China
b University of Chinese Academy of Sciences, Beijing 100049, China

A R T I C L E   I N F O

Article history:
Received 11 June 2018
Received in revised form 12 September 2018
Accepted 16 September 2018
Available online 17 September 2018

Keywords:
Chitosan
Quaternary
Antifungal

ABSTRACT

Chemical modification is one of the prominent methods used to improve the water solubility and bioactivity of chitosan. In this paper, a series of quaternary ammonium chitosan derivatives based on 6-O-chloroacetylated chitosan (CAClC) were successfully designed and synthesized. Detailed structural characterization was carried out by means of FT-IR, 1H NMR spectroscopy, and elemental analysis. Furthermore, the antifungal activity against Botrytis cinerea, Gibberella zeae, and Physalospora piricola Nose was estimated using in vitro hypha measurements. Most of the quaternary ammonium chitosan derivatives showed an inhibitory index of ~90% at 1.0 mg/mL and exhibited enhanced antifungal activity when compared to chitosan. On one hand, the higher density of positive charge contributed to the antifungal action. In addition, the inhibitory activity decreased roughly in the order of TrIM [(CH3)2(CH2CH2CH2CH3)] > TrIE [(CH3)3CHCH2] > TrIP [(CH3)2CH2CH2CH3] > TrIB [(CH3)2CH2CH2CH3]; NTNE [(CH3)2(CH2CH3)] > NNTB [(CH3)2(CH2CH2CH2CH3)] > NTK [(CH3)2(CH2CH2CH3)] at 1.0 mg/mL. The antifungal properties of all the quaternary ammonium chitosan derivatives against the targeted fungi decreased upon increasing the alkyl chain length.

© 2018 Published by Elsevier B.V.

1. Introduction

There are large crop yield losses worldwide every year, especially in developing countries, that are mainly caused by plant pathogenic fungi-derived agricultural diseases [1]. For example, Botrytis cinerea is the pathogen that causes fruits and vegetables to rot shortly after harvest, which causes a considerable post-harvest economic loss [2]. Gibberella zeae is a prominent pathogen in cereals such as wheat, barley and maize, which produces mycotoxins that are harmful to animals and humans [3]. Physalospora piricola Nose is a fungal pathogen in pear, apple, begonia, and other fruit trees, which causes serious damage resulting in rotten fruit and dead branches [4]. The chemical control of pathogens is responsible for the increased productivity and quality of crops [5,6]. However, an inappropriate and non-discriminatory use of these chemical fungicides has resulted in serious ecological, environmental, and health problems [7]. Therefore, the awareness of developing safe, efficacious, and environmentally friendly natural alternatives for plant protection and preservation purposes is increasing [8].

Chitosan, with nontoxic and biodegradable properties, has recently gained more and more attention [9]. Chitosan [poly(1,4-β-d-glucopyranosamine)], as the only cationic polysaccharide currently found in nature, is generally prepared via the deacetylation of chitin, which is available in the skeletal materials of crustaceans and insects, and the cell walls of bacteria and fungi [10]. As one of the most abundant natural polysaccharides (after cellulose) found on the earth, chitosan has several extraordinary advantages, including nontoxicity, biodegradability, and biocompatibility [9–12]. As a kind of natural renewable resource, chitosan has been applied in food, pharmaceuticals, beverages, papermaking, packaging, and textiles [13–18]. However, the widespread application of chitosan has been limited due to its high degree of polymerization and poor water solubility in neutral and alkaline conditions [19]. Chemical modification is one of the most effective ways to improve the water solubility and bioactivity of chitosan [20]. To date, a large number of chitosan derivatives have been synthesized via quaternization, carboxylation, phosphorylation, and sulfation to improve the water solubility and the bioactivity of chitosan [21–24]. Therefore, it is highly desirable to develop chitosan derivatives with high bioactivity and good water solubility via chemical modification.

It has been reported that molecules containing quaternized amine moieties, such as benzalkonium, pyridinium, and triethylammonium bromide, have prominent antibacterial, antifungal, and antiprotozoal properties [25,26]. Moreover, quaternary ammonium salts have been
widely applied in bandages, the solutions used to wash open wounds and preoperative disinfectants due to their low toxicity [27]. Based on the superposition principle, many kinds of polysaccharide polymers such as starch, inulin, and chitosan have been quarternized to combine the properties of the ammonium group with the intrinsic properties of the corresponding polymer [28–30]. The quaternary ammonium chitosan derivatives exhibit enhanced inhibitory properties when compared with the Schiff base derivatives of chitosan and N-substituted chitosan [31]. The higher density of positive charge will improve the antifungal activity. In addition, the length of the alkyl substituents at the periphery of the polymer can affect the antifungal activity of chitosan derivatives [32]. The antifungal activity of the chitosan derivatives increases upon increasing the length of the alkyl substituents [33]. This observation has inspired us to quaternize chitosan using a tertiary amine with different alkyl chain lengths based on 6-O-chloroacetylated chitosan. The chemical structures of the chitosan derivatives were characterized using FT-IR, 1H NMR spectroscopy, and elemental analysis. Furthermore, three kinds of common fungi in agriculture including Botrytis cinerea, Gibberella zeae, and Physalospora piricola Nose were investigated in this paper to evaluate the antifungal activity of the quaternary ammonium chitosan derivatives.

2. Experimental

2.1. Materials

Chitosan (MW 100.0–200.0 kDa, the degree of deacetylation 81.5%) was purchased from Introduction of Jinhu Crust Product Co., LTD (China). Trimethylamine, Triethylamine, N,N-Dipropyl-1-propanamine, Tributylamine, N,N-Dimethylthethylamine, N,N-Dimethylaminobutane, N,N-Dimethyldecylamine, chloroacetyl chloride, acetone, N,N-dimethylformamide (DMF), N-methyl-2-pyrrolidone (NMP), iodomethane, sodium iodide, ethyl ether, and sodium hydroxide were purchased from Sinopharm Chemical Reagent Co., Ltd. The other reagents were all analytical grades and were used without further purification.

2.2. Analytical methods

FT-IR spectrometers were recorded on a Jasco-4100 ranging from 4000 to 400 cm⁻¹ (Japan, provided by JASCO Co., Ltd., Shanghai, China) at 25 °C with KBr disks. 1H NMR spectra were recorded on a Bruker AVIII 500 spectrometer (Fällanden, Switzerland, provided by Bruker Biospin CN/Bruker (Beijing) Tech. and Serv. Co., Ltd., Beijing, China), using D₂O, or CH₃COOD as solvents with tetramethysilane (TMS) as internal standard. Chemical shift values were given in δ (ppm). The elemental analyses (C, H, and N) were performed on a Vario EL III (Elementar, Germany). The Degree of Substitution (DS) of chitosan derivatives were calculated based on the percentages of carbon and nitrogen. The results were processed and reported as mean ± SD by computer programs Excel (Microsoft, Redmond), OriginLab Pro 8 (OriginLab, Northampton, MA, USA), and MestReNova (Mestrelab Research S.L.).

2.3. The synthesis of the chitosan derivatives

2.3.1. The synthesis of 6-O-chloroacetyl-2,N,N-trimethyl quaternary ammonium chitosan derivative (CACIC)

CACIC was synthesized as follows in Scheme 1. 0.6 g (4.0 mmol) chitosan was well dispersed in 50.0 mL NMP for 1 h at room temperature. Then 6.0 mL (22.0 mmol) aqueous sodium hydroxide solution (15.0%, w/t), 1.8 g (12.0 mmol) sodium iodide, and 6.0 mL (96.0 mmol) iodomethane were added, and the reaction was carried out with stirring for 1 h at 60 °C. The product was isolated by pouring the reaction solution into excess ethanol. The precipitate was collected by filtration and washed with ethanol. Then the precipitate was dispersed into 20.0 mL DMF, and 3.0 mL (37.0 mmol) chloroacetyl chloride was added into the solution. After stirring 24 h at 30 °C, the reaction was continued to carry out for 6 h at 60 °C. The solution was precipitated by the addition of excess ethanol. The precipitate was filtrated and washed with ethanol. After being diazylized against deionized water for 2 days, the product CACIC was obtained by freeze-drying. Yield: 80.4%; DS: 65.5% (Table 1).

2.3.2. The synthesis of quaternary ammonium chitosan derivatives

0.6 g (2.0 mmol) CACIC were dispersed into 20.0 mL DMF into a 100.0 mL round-bottom flask. Then 6.0 mmol all kinds of tertiary amine products were dropped wisely with stirring respectively. The unreactive reagents were extracted in Soxhlet Apparatus with the mixture of ethanol and acetone for 48 h. The products (TriM, TriE, TriP, TriB, NNTE, NNTB, and NNTK) were obtained by the freeze-drying overnight in vacuum. TriM: Yield: 70.6%; DS: 84.3%; TriE: Yield: 70.9%; DS: 77.7%; TriP: Yield: 74.2%; DS: 78.2%; TriB: Yield: 80.3%; DS: 68.3%; NNTE: Yield: 76.5%; DS: 63.0%; NNTB: Yield: 74.3%; DS: 66.3%; NNTK: Yield: 73.6%; DS: 45.3% (Table 1).

2.4. Antifungal assay

Antifungal assay was evaluated against Botrytis cinerea, Gibberella zeae, and Physalospora piricola Nose in vitro by measuring the growth rate of mycelium according to the method of Guo et al. [31] Briefly,
the compounds (chitosan and all chitosan derivatives) were dissolved in distilled water at a concentration of 5.0 mg/mL at room temperature. Then, the test sample solution was added to the sterilized potato dextrose agar (PDA) medium to get a final concentration of 0.1, 0.5, and 1.0 mg/mL respectively, and poured into the sterilized Petri dishes (9.0 cm). Identical volume distilled water substituting samples were poured into control plates. Finally, the fungi mycelia disk with a diameter of 5.0 mm was placed into the center of the PDA Petri dishes and incubated at 27 °C for 2–3 days. When the diameter of the fungi mycelium reached to the edges of the control plate (without the sample), the inhibitory index was calculated as follows:

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

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. The experiments are performed three times. And all the data are averaged and expressed as means ± SD (\(n = 3\)).

The Scheffe’s multiple range test, a single-step multiple comparison procedure in analysis of variance, which was applied to the set of estimates of all possible contrasts among the factor level means, was used to evaluate the inhibitory indices differences in antifungal tests. The level of \(P < 0.05\) was considered statistically significantly.

3. Results and discussion

3.1. Structure of the chitosan derivatives

The FT-IR spectra recorded for chitosan and chitosan derivatives are presented in Fig. 1. The spectrum of chitosan shows that the saccharide characteristic bands: \(\nu (\text{O-H})\) or \(\nu (\text{N-H})\) at 3428 cm\(^{-1}\), \(\nu (\text{C-H})\) at 2919 cm\(^{-1}\), \(\nu (\text{amide I band})\) at 1643 cm\(^{-1}\), \(\delta (\text{C-H})\) at 1427 at 1380 cm\(^{-1}\), \(\nu (\text{amide III band})\) at 1322 cm\(^{-1}\), \(\delta (\text{O-H})\) at 1261 cm\(^{-1}\), \(\nu (\text{C-O})\) at 1068 cm\(^{-1}\), and the \(\beta\) glycosidic bond at 898 cm\(^{-1}\). New peaks at about 1747 cm\(^{-1}\), 1469 cm\(^{-1}\), and 794 cm\(^{-1}\) were observed in the spectrum recorded for CACIC, which were assigned to \(-\text{C}=	ext{O}, -\text{N}^+ (\text{CH}_3)_3\), and \(-\text{C}=	ext{Cl}\), respectively. All the data confirm the formation of the chloroacetyl chitosan derivative. After the chemical reaction between the chloroacetyl chitosan derivatives and the tertiary amine, the new peak observed at about 1481 cm\(^{-1}\) was assigned to the new quaternary ammonium salts, TriM, TriE, TriP, TriB, NNTE, NNTB, and NNTK, respectively. Meanwhile, the peak observed at about 1751 cm\(^{-1}\) disappeared, which illustrated that the band corresponding to \(-\text{C}=	ext{Cl}\) was destroyed. The above mentioned results demonstrated that the quaternary ammonium chitosan derivatives were obtained.

Fig. 2 shows the \(^1\text{H NMR}\) spectra recorded for the quaternary ammonium chitosan derivatives. It is known that the single peak at \(\delta = 3.0\) ppm, multiple peaks at \(\delta = 3.6–3.9\) ppm and the peak at \(\delta = 4.6\) ppm can be assigned to the hydrogen protons at C-2, C-3 to C-6, and C-1, respectively [34]. Meanwhile, the peak at 2.0 ppm reveals the presence of the hydrogen protons in the N-acetyl residue [35]. In addition, the molecular proton signals derived from CACIC could be observed distinctly after quaternization with the various tertiary amines studied, such as the characteristic resonance peak of \(-N^+ (\text{CH}_3)_3\) at \(\delta = 3.3\) ppm. Besides, the methylene protons of the \(-\text{COCH}_2\text{Cl}\) group shows a new resonance peak at \(\delta = 4.3\) ppm. In addition, the characteristic proton signals derived from CACIC could be observed distinctly after quaternization with the various tertiary amines studied, such as the characteristic resonance of \(-\text{COCH}_2\text{Cl}\) at \(\delta = 4.3\) ppm was greatly weakened. Besides, the new peak at \(\delta = 3.2\) or 3.1 ppm was assigned to the new quaternary ammonium salts formed by the tertiary amine in the spectra recorded for TriM, TriE, TriP, TriB, NNTE, NNTB, and NNTK, respectively. Furthermore, there were new peaks observed at \(\delta = 0.8–1.8\) ppm that could be assigned to the alkyl chains with different alkyl chain lengths, which are marked in Fig. 2. The abovementioned results further demonstrated that the quaternary ammonium chitosan derivatives were obtained.

3.2. Solubility and antifungal activity

Fig. 3 shows the aqueous solution of the as-synthesized quaternary ammonium chitosan derivatives and chitosan at 1.0 mg/mL. It was obvious that chitosan with a molecular weight 100.0–200.0 kDa had a poor water solubility in neutral water. The introduction of the quaternary ammonium salts promoted the water solubility of chitosan. The quaternary ammonium salts as hydrophilic moieties can remarkably improve the solubility of chitosan [36]. The TriM, TriE, TriP, TriB, NNTE, NNTB, and NNTK products showed favorable water solubility and their solutions were prepared at a concentration of 0.1–1.0 mg/mL at room temperature. In view of the low solubility of chitosan in water, the water-soluble chitosan with a molecular weight 8.0 kDa was chosen to study the antifungal activity in this paper.

### Table 1

| Compounds   | Yields (%) | Elemental analyses (%) | Degrees of substitution (%) | Deacetylation (%) |
|-------------|------------|------------------------|-----------------------------|-------------------|
|             |            | C          | N          | C/N             |                |
| Chitosan    | 40.86      | 7.47       | 5.47       | 81.5            |                |
| CACIC       | 80.4       | 37.46      | 4.36       | 8.60            | 65.5            |
| TriM        | 70.6       | 38.21      | 6.14       | 6.22            | 84.3            |
| TriE        | 70.9       | 36.87      | 4.95       | 7.45            | 77.7            |
| TriP        | 74.2       | 42.07      | 4.91       | 8.57            | 78.2            |
| TriB        | 80.3       | 37.90      | 3.94       | 9.62            | 68.3            |
| NNTE        | 76.5       | 39.08      | 5.65       | 6.92            | 63.0            |
| NNTB        | 74.3       | 42.69      | 5.66       | 7.55            | 66.3            |
| NNTK        | 73.6       | 47.56      | 5.07       | 9.36            | 45.3            |
The elemental analyses, yields and degree of substitution of the chitosan ammonium salts are shown in Table 1. The antifungal activities of chitosan and the chitosan derivatives against three common plant-threatening fungi, Botrytis cinerea, Gibberella zeae, and Physalospora piricola Nose are shown in Figs. 4–6.

As shown in Fig. 4, chitosan and all the quaternary ammonium chitosan derivatives displayed antifungal activity against Botrytis cinerea at all the tested concentrations. Meantime, the antifungal indices of all the samples were enhanced upon increasing the concentration. When compared with the water-soluble chitosan and the intermediate product CACIC with an inhibitory index of 23.8% and 28.9%, respectively, all the quaternary ammonium chitosan derivatives (TriB, 63.3%, TriP, 91.8%, TriE 97.0%, TriM 98.0%, NNTE 98.9%, NNTB 96.4%, NNTK 94.6%) had enhanced antifungal activity at 1.0 mg/mL, which was in
in accordance with the conclusion that the higher positive charge density in quaternary ammonium chitosan derivatives can contribute to the antifungal activity [37]. The positive charge can interact with some of the anionic components found on the cell surface, such as glucan, mannan, proteins, and lipids, to form an impervious layer around the cell. On one hand, the impervious layer may prevent essential nutrients from entering the cell. On the other hand, this interaction may damage the cell wall, which will cause cell death because of the leakage of cell constituents [38]. Moreover, based on a “permeability” point of view that the oil film outside the cell wall will only allow lipid soluble substances to pass, the lipophilic characteristics of the alkyl chain may be another reason to explain the mechanism of the increased antifungal activity. In addition, the effects of the different lengths of the alkyl chains on the antifungal activity were also evaluated in this paper. As illustrated by the data, the antifungal activities of all the chitosan derivatives were similar at the lower concentration. However, upon increasing the concentration of the samples, the antifungal activity decreased with the increasing length of the alkyl chains in the order of TriM > TriE > TriP > TriB; NNTB > NNTK > NNT. It was reasonable to interpret that the longer alkyl chains with stronger electron-donating abilities tended to donate more electrons to the quaternary ammonium, which decreased the density of the positive charge of the quaternary ammonium and eventually led to a reduction in the antifungal activity.

Besides, the inhibitory indices of chitosan and the target products against Gibberella zeae and Physalospora piricola Nose are shown in Figs. 5 and 6 respectively, and were almost identical to the antifungal activity against Botrytis cinerea. All samples showed antifungal activity against Gibberella zeae and Physalospora piricola Nose. In addition, all samples were mounted up with an increasing concentration. All quaternary ammonium salt chitosan derivatives showed enhanced antifungal activity when compared with chitosan and the intermediate product CACIC against the targeted microorganisms. The inhibitory indices of chitosan, CACIC, TriB, TriP, TriE, TriM, NNTB, and NNTK were 37.5%, 37.9%, 60.4%, 66.7%, 89.8%, 90.5%, 90.2%, 87.7%, and 86.1%, respectively at 1.0 mg/mL against Gibberella zeae. Upon increasing the length of the alkyl chain, the antifungal activity decreased in the order of TriM > TriE > TriP > TriB; NNTB > NNTK at 1.0 mg/mL, which was similar to the antifungal activity observed against Botrytis cinerea. Moreover, the inhibitory indices of chitosan, CACIC, TriB, TriP, TriE, TriM, NNTB, and NNTK were 35.9%, 40.2%, 91.5%, 91.6%, 96.8%, 100%, 96.6%, 96.5%, and 94.1%, respectively at 1.0 mg/mL against Physalospora piricola Nose. As illustrated by the data, TriM could almost completely inhibit the growth of Physalospora piricola Nose, especially at 1.0 mg/mL. Besides, the antifungal activity decreased upon increasing the length of the alkyl chain in the order of TriM > TriE > TriP > TriB; NNTB > NNTK at 1.0 mg/mL. The results further confirmed that the quaternary ammonium chitosan derivatives significantly contributing to the antifungal activity, and the length of the alkyl chain had an important role in the antifungal activity.

4. Conclusions

In this study, a series of quaternary ammonium chitosan derivatives were successfully designed, synthesized, and characterized using FT-IR and $^1$H NMR spectroscopy. The antifungal activities against three kinds of phytopathogens including Botrytis cinerea, Gibberella zeae, and Physalospora piricola Nose were estimated using in vitro hyphal measurements. All quaternary ammonium chitosan derivatives had good solubility in water and exhibited higher inhibitory indices than chitosan and the intermediate product CACIC. On one hand, the higher density of the positive charge contributed to the antifungal activity against all the targeted microorganisms. On the other hand, the alkyl chains with lipophilic characteristics easily pass the oil film outside the cell wall based on the theory of permeability, which may be another reason for the improvement in the antifungal activity of the quaternary ammonium chitosan derivatives. Moreover, the length of the alkyl chains was another factor that affected the antifungal activity of the quaternary ammonium chitosan derivatives with the antifungal properties against the targeted fungi decreasing upon increasing the alkyl chain length. The products described in this paper have great potential as alternatives to some harmful pesticides used for plant disease control.

Acknowledgements

We thank the National Natural Science Foundation of China (41576156), Natural Science Foundation of Shandong Province of China (ZR2017BD015), and Yantai Science and Technology Development Plan (2015ZH078), and Technology Research Funds Projects of Ocean (No. 2015418022-3) for financial support of this work.

Conflict of interest statement

The authors have declared no conflicts of interest.
Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.ijbiomac.2018.09.099.

References

[1] W. Liu, Y. Qin, S. Liu, R. Xing, H. Yu, X. Chen, K. Li, P. Li, Int. J. Biol. Macromol. 114 (2018) 942–949.
[2] Y. Li, X. Shao, J. Xu, Y. Wei, F. Xu, H. Wang, Food Chem. 234 (2017) 62–67.
[3] J. Zhang, W. Tan, Z. Zhang, Y. Song, Q. Li, F. Dong, Z. Guo, Int. J. Biol. Macromol. 109 (2018) 1061–1067.
[4] X. Meng, L. Yang, J.F. Kennedy, S. Tian, Carbohydr. Polym. 81 (2010) 70–75.
[5] D.-M.-C. Nguyen, D.-J. Seo, H.-B. Lee, I.-S. Kim, K.-Y. Kim, R.-D. Park, W.-J. Jung, Microb. Pathog. 56 (2013) 8–15.
[6] J. Breger, B.B. Fuchs, C. Aperis, T.I. Moy, F.M. Ausubel, E. Mylonakis, PLoS Pathog. 3 (2007) e0618-0178.
[7] B. Yuan, P.-Y. Xu, Y.-J. Zhang, P.-P. Wang, H. Yu, J.-H. Jiang, Int. J. Biol. Macromol. 66 (2014) 7–14.
[8] A. Gotor-Vila, N. Teixidó, A. Di Francesco, J. USall, L. Ugolini, R. Torres, M. Mari, Food Microbiol. 64 (2017) 219–225.
[9] W. Sajomsang, P. Gonil, S. Saesoo, C. Ovatlarnporn, Int. J. Biol. Macromol. 50 (2012) 263–269.
[10] Z. Guo, R. Xing, S. Liu, Z. Zhong, X. Ji, L. Wang, P. Li, Carbohydr. Polym. 71 (2008) 694–697.
[11] Z. Guo, H. Liu, XX. Ji, P. Li, Bioorg. Med. Chem. Lett. 16 (2006) 6348–6350.
[12] Q. Li, W. Tan, C. Zhang, C. Gu, Z. Guo, Int. J. Biol. Macromol. 91 (2016) 623–629.
[13] P. Chantarasataporn, P. Tepkasikul, Y. Kingcha, R. Yoksan, R. Pichyangkura, W. Visessanguan, S. Charichanchai, Food Chem. 159 (2015) 463–470.
[14] M.N. Kumar, R.A. Muzzarelli, C. Muzzarelli, H. Sashiwa, A.J. Domb, Carbohydr. Polym. 342 (2007) 1329–1332.
[15] Q. Li, J. Ren, F. Dong, Y. Feng, G. Gu, Z. Guo, Carbohydr. Res. 373 (2013) 103–107.
[16] B.M.E.I., J. Appl. Polym. Sci. 117 (2010) 3960–3968.
[17] L. Wei et al. / International Journal of Biological Macromolecules 129 (2019) 1127–1132