Synthesis of chitosan derivative with diethylidithiocarbamate and its antifungal activity

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With an aim to discover novel chitosan derivatives with enhanced antifungal properties compared with chitosan, diethylidithiocarbamate chitosan (EtDTCCS) was investigated and its structure was well identified. The antifungal activity of EtDTCCS against Alternaria porri (A. porri), Gloeosporium theae sinensis Miyake (G. theae sinensis), and Stemphylium solani Weber (S. solani) was tested at 0.25, 0.5, and 1.0 mg/mL, respectively. Compared with plain chitosan, EtDTCCS shows better inhibitory effect with 93.2\% inhibitory index on G. theae sinensis at 1.0 mg/mL, even stronger than for polyoxin (82.5\%). It was inferred derivatives of this kind may find potential applications for the treatment of various crop-threatening diseases.

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1. Introduction

Chitosan, (1→4)-2-amino-2-deoxy-β-d-glucan, the cationic deacetylated derivative of chitin, is a well-confirmed biopolymer owing to biocompatibility, biodegradability and absence of toxicity [1]. Among its widespread biological activities, antimicrobial activity has attracted extensive attention. It is proved that chitosan has a broad-spectrum antimicrobial activity against a variety of bacteria and fungi [2]. However, the weak antimicrobial properties have hampered its widespread application.

Chemical modification of chitosan to obtain new bioactive derivatives is of interest because such procedure may not change the fundamental skeleton of chitosan [3–5]. Additionally, the new generated chitosan derivatives may also possess original properties of the group introduced. In fact, the strategies for introducing functional groups into chitosan are widely used. These grafting techniques involve mainly condensation reactions such as alkylation [6], acylation [7], sulfonation [8], and so on. For example, Muzzarelli et al. reported antimicrobial properties of N-carboxybutyl (NCB) chitosan tested against 298 microbial strains, the data displayed NCB-chitosan may have a potential application as wound dressing [9]. Rabea and his co-authors reported the preparation and antifungal properties of N-alkyl chitosan (NAC) derivatives [10]. The results showed most of the NAC derivatives exerted better antifungal activity than original chitosan. Eweis et al. described antifungal activity of a chitosan thiourea derivative (TUCS) against Rhizoctonia solani, Sclerotium rolfsii, and Fusarium solani. The prepared chitosan derivative had a significant inhibitory effect on the investigated fungi at the concentrations of 5–1000 mg/mL [11].

It is notable that dithiocarbamates modified chitosan and its applications have gained considerable attention in the past few years [12,13]. Chitosan dithiocarbamate is a chitosan derivative in which C2 amino groups are substituted by dithiocarbamate groups. It is usually achieved by adding carbon disulfide (CS\textsubscript{2}) and alkaline dissolved in alcohol. Owing to the strong metal binding capacity of dithiocarbamates, various dithiocarbamate chitosan derivatives have been prepared and investigated [14,15]. However, current applications of chitosan dithiocarbamates were mainly focused on metal chelating properties and these chitosan derivatives were usually obtained via insertion of carbon disulfide to form dithiocarbamate directly. There were few articles involved antimicrobial properties or additional synthetic methods of chitosan dithiocarbamates. In fact, dithiocarbamates are a class of fungicides extensively used for the control of a large variety of diseases affecting several types of crops [16–18].

In view of the potential antimicrobial applications of chitosan dithiocarbamates, this study aims to extend synthetic methods and applications of chitosan dithiocarbamates. Hence, diethyl
dithiocarbamate chitosan was synthesized and its antifungal activity against some common plant pathogenic fungi was investigated.

2. Experiment

2.1. Materials

Chitosan, [weight-average molecular weight (MW) of 230 kDa, with a degree of deacetylation of 0.87], was purchased from Qingdao Baicheng Biochemical Corp., Shandong, China. Polyoxin, 10% wettable powders, was obtained from Kaken Pharmaceutical Co., Ltd. Diethyamine, carbon disulfide, and chloroacetyl chloride were purchased from Sinopharm Group Chemical Reagent Co. Ltd. (China). Other commercial chemical reagents used in the experiment were all analytical grade. Double-distilled water was used throughout the experiment.

2.2. Microbial strains

Three plant pathogenic fungi, Alternaria porri (A. porri), Gloeosporium theae sinensis Miyake (G. theae sinensis), and Stemphylium solani Weber (S. solani), were used as the tested microorganisms. They were obtained from Qingdao Academy of Agricultural Sciences. Fungi for testing were stored on PDA medium at 4 °C.

2.3. Analytical conditions

Fourier transform infrared (FT-IR) spectra of the derivatives were obtained on a Thermo Scientific Nicolet iS10 FT-IR spectrometer between 4000 and 400 cm⁻¹ regions using KBr discs. The 13C NMR (Internal standard: acetone) spectra of the polymers were recorded on a JEDL JNM-ECP600 spectrometer, using CDCl3 and D2O as solvents. The elemental analysis (C, H, N, and S) was determined according to a Vario EL-III elemental analyzer and the content of carbon, hydrogen, nitrogen, and sulfur were estimated. The degree of substitution (DS) of diethyl dithiocarbamate chitosan (EtDTCCS) was calculated by the percentages of sulfur. DSC temperature scan was performed using The Pyris Diamond DSC (Perkin Elmer). The samples were sealed in aluminum pans and heated from 50 °C to 300 °C under 10 °C/min under the protection of nitrogen purge gas. An empty pan was used as a reference in the test. Three tests were performed for each sample. The gross morphology and microstructures of the samples was observed under scanning electron microscopy by using KYKY-2800B SEM.

2.4. Synthesis of diethyl dithiocarbamate chitosan (EtDTCCS)

2.4.1. Synthesis of potassium dimethyl dithiocarbamate

A mixture of diethyamine (100 mmol, 7.3 g) and potassium hydroxide (100 mmol, 5.6 g) was stirred in a 250 mL round bottomed flask with 100 mL methanol for an hour at room temperature. Then carbon disulfide (100 mmol, 7.6 g) was added drop-wise over 30 min to the mixture and the solution was stirred for another 10 h. Next, the solvent was removed under vacuum to give a crude product, and then the product was recrystallized two times from ethanol to obtain pure potassium dimethyl dithiocarbamate as a yellow solid, 87.1% yield. 1H NMR (600 MHz, DMSO-d6, δ): 3.96 (q, 2H, CH3), 1.07 (t, 3H, CH3).

2.4.2. Preparation of diethyl dithiocarbamate chitosan (EtDTCCS)

Chloroacetyl chitosan (CACS) was prepared according to the literatures described by Zhong [19]. Then chloroacetyl chitosan (10 mmol) was mixed with dimethyl dithiocarbamate (10 mmol) in double-distilled water (50 mL). After reacted for 8 h at 80 °C, the resultant was cooled to room temperature and filtered through Filter Funnel Buchner. The precipitate was washed with ethanol and dried to give diethyl dithiocarbamate chitosan (Scheme 1), yield: 56.5%, DS: 0.354.

2.5. Antifungal bioassays

Antifungal assay was evaluated against Alternaria porri (A. porri), Gloeosporium theae sinensis Miyake (G. theae sinensis), and Stemphylium solani Weber (S. solani) in vitro by mycelium growth rate test according to the literatures [20,21]. The tested concentration was 0.25 mg/mL, 0.5 mg/mL, and 1.0 mg/L, respectively. Each experiment was performed in three replicates, and the data were averaged. Results with P<0.05 were considered statistically significant.

3. Results and discussion

3.1. Preparation and characterization of diethyl dithiocarbamate chitosan (EtDTCCS)

In our attempt to obtain new dithiocarbamate chitosan derivatives with potential antifungal activity, diethyl dithiocarbamate chitosan (EtDTCCS) was synthesized, purified and its structure was confirmed from their analytical and spectral data.

Fig. 1 presents the infrared transmittance spectra for the prepared samples. For chitosan, the broad band around 3400 cm⁻¹ attributed to –OH and –NH stretching vibration. The weak peak at 2875 cm⁻¹ was the characteristic absorbance of –CH. The absorption peak at 1592 cm⁻¹ is associated with NH2 bending vibration. Additionally, the absorption peaks assigned to symmetric stretching of the C=O–C were observed at 1157 cm⁻¹, 1080 cm⁻¹ and 1021 cm⁻¹. Compared with chitosan, new bands at 1643 cm⁻¹ (the amide I (C=O), 1533 cm⁻¹ (the amide II, NH–C=O), 1380 cm⁻¹ (C–N) were observed for chloroacetylated chitosan (CACS). In addition, the peak at 1592 cm⁻¹ of the primary amine disappeared which meant amino has been substituted. These results also coincided with that reported before [19]. All of the results exhibited CACS had been successfully obtained.

In the spectrum of diethyl dithiocarbamate chitosan (EtDTCCS), new peaks appear at 2879 cm⁻¹ (S=CH2), 1423 cm⁻¹ (NH–C=S), 1261 cm⁻¹ (C=S). Additionally, the amide I stretching peak has shifted from 1643 cm⁻¹ to 1673 cm⁻¹ due to the introduction of dithiocarbamate group. Hence, the FT-IR data are indicative of

![Image](348x122 to 589x321)

Fig. 1. FT-IR spectra of chitosan, chloroacetylated chitosan (CACS), and diethyl dithiocarbamate chitosan (EtDTCCS).
successful synthesis of the chitosan derivative and support the proposed reaction.

The structures of chitosan and its derivatives were further confirmed by $^{13}$C NMR. As seen in Fig. 2, the signals for chitosan can be well separated and identified. δ: 21.5 (Ac–C), 55.4 (C2), 59.6 (C6), 70.4 (C3), 76.8 (C4), 97.1 (C1), 175.3 (C=O). These spectra of chitosan coincided with that reported before [22,23]. For CACS, new peaks were observed at 100.8 (CH2Cl) and 187.9 (Cl–C=O), respectively. It was inferred that chloroethyl groups have been grafted on chitosan backbone.

For diethyl dithiocarbamate chitosan (EtDTCCS), it was obviously observed that new strong peaks appear at 10.6 ppm (CH3) and 42.9 ppm (CH2CH3). In addition, the new peak at δ = 176.9 ppm was assigned to dithiocarbamate group (SCH2C=O). Additionally,
The gross morphology and microstructures of original chitosan, chloroacetylated chitosan (CACS), and diethyl dithiocarbamate chitosan (EtDTCCS) was observed under scanning electron microscopy (Fig. 4). It was noticed that chitosan and CACS display nonporous and flat phase surface, while EtDTCCS exhibit the highly porous interconnectivity. This result also accorded with above results and indicated the change of microstructures of chitosan via chemical modification.

3.2. Antifungal activity of diethyl dithiocarbamate chitosan (EtDTCCS)

It was proved that antifungal properties of chitosan and its derivatives are influenced by various factors such as molecular weight [26], pH value [2] and so on. In this study, three crop-threatening pathogenic fungi: Alternaria porri (A. porri), Gloeosporium theae sinensis Miyake (G. theae sinensis), and Stemphylium solani Weber (S. solani) were selected to evaluate antifungal activity of EtDTCCS in acid medium. Hence, the presence of the acetic acid may contribute to the antifungal action. So the inhibitory effect of various concentrations of acetic acid aqueous solution on the tested fungal strains corresponding to the investigated concentrations was evaluated. As shown in Fig. 5, the inhibitory effect of acetic acid on the selected fungi was influenced by the concentrations. At low concentrations, no higher than 0.05% (corresponding to concentrations of 1.0 mg/mL of the samples), there was no or slight inhibitory effect for acetic acid aqueous solution on the fungi. That is to say, at the tested concentrations ranged from 0.25 to 1.0 mg/mL, the diluted acetic acid had no contribution to antifungal activity.

The antifungal results of diethyl dithiocarbamate chitosan (EtDTCCS) against the fungi were shown in Table 1. In general, the chitosan derivative exhibited significant inhibitory effect on the fungi especially G. theae sinensis.

A. porri can cause onion purple blotch disease, which leads to serious losses in onion. In previous study, we have prepared
ammonium dithiocarbamate chitosan (ADTCCS) and triethylene diamine dithiocarbamate chitosan (TEDADTCCS) to evaluate their antifungal properties on *A. porri*. Antifungal results displayed both TEDADTCCS and ADTCCS had no obviously enhanced antifungal activity compared with chitosan [13]. In the present study, inhibitory effect of the new obtained dithiocarbamate chitosan derivative on *A. porri* was also tested. As shown in Table 1, EtDTCCS displayed stronger inhibitory effect than original chitosan, and the antifungal activity enhanced with increasing concentration. Antifungal index of the derivative ranged from 47.7% to 88.4%, while the index of chitosan ranged from 0% to 65.1%. It was concluded that antifungal activity of chitosan enhanced obviously via chemical modification. However, antifungal activity of EtDTCCS against *A. porri* cannot compare with that of polyoxin whose max antifungal index reached 100%.

*Gloeosporium theae sinensis* Miyake (*G. theae sinensis*) is a pathogen which causes anthracnose on tea plants. The disease causes death of crops with great economic losses. As shown in Table 1 and Fig. 6, the antifungal index of the derivative ranged from 38.0% to 93.2%. It is interesting to notice that EtDTCCS 93.2% inhibited growth of the mycelium at higher concentration of 1.0 mg/mL, even stronger than polyoxin whose antifungal index was 82.5%. It was deduced the fungus was more sensitive than *A. porri* to EtDTCCS.

*S. solani*, a common crop-threatening fungus, has become one of the most serious diseases to tomato in some regions. Table 1
Presented antifungal results of EtDTCCS against S. solani. Compared with chitosan, EtDTCCS exhibited slightly better antifungal activity. The inhibitory index of the derivatives ranged from 46.0% to 77.8%, while antifungal index of chitosan was in the range of 12.3–50.9%. However, antifungal activity of the chitosan derivative was not as strong as polyixin. It was shown that chitosan exhibited excellent antifungal properties against S. solani. At lower concentration of 0.25 mg/mL, it even 100% inhibited growth of the mycelium.

### 4. Conclusion

In this study, diethyl dithiocarbamate chitosan (EtDTCCS) were prepared and its structure was well defined. Additionally, its antifungal activity was evaluated. In general, the chitosan derivatives obviously inhibited the investigated fungi compared with chitosan. In addition, antifungal activity of EtDTCCS against G. theae sinensis was comparable to polyixin. At higher concentration of 1.0 mg/mL, EtDTCCS even presented stronger antifungal properties than polyoxin. However, its activity was weaker than the positive control polyoxin in general. It was inferred that antifungal activity of chitosan enhanced due to introduction of antifungal group to chitosan chain. It is important to notice that derivatives of this class might serve as new leading structures for further design of antifungal agents.

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