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Preparation, characterization and antimicrobial activity of chitosan–Zn complex

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Abstract

Five chitosan–zinc complexes with different zinc content were prepared and characterized by FT-IR, XRD, AAS and elemental analysis. It was found that the complexes with different zinc content had different molecule structure. Three possible structures corresponding to different chelate ratios were proposed and preliminarily complexation mechanisms were discussed. In vitro antimicrobial activities of complexes were evaluated against 11 species of bacteria and fungi. The complexes showed wide spectrum of effective antimicrobial activities, which were 2–8 times and 4–16 times higher than those of chitosan and zinc sulfate, respectively, and improved with increasing content of zinc ions. The complexes had a better antibacterial activity than antifungal activity, and showed excellent activity particularly against *E. Coli*. and Corynebacterial both with a MIC value of 0.000313% (CS–Zn w/v).

Keywords: Chitosan–Zn; Complexes; Antimicrobial activity

1. Introduction

Chitin, commonly found in the exoskeleton or cuticles of many invertebrates and in the cell walls of most fungi and some algae, is one of the most abundant, easily obtained, and renewable natural polymers, second only to cellulose. Chitosan (CS) is a deacetylated derivative of chitin, in which there are a great deal of amine groups and hydroxyl groups. Such structure moods bring CS tremendous ability to form metal complexes (Juang, Wu, & Tsang, 1999; Delben, Stefancich, & Muzzarelli, 1992; Onsoyen & Skaugrud, 1990).

Zinc (Zn) is one of the metal ions that are easiest to coordinate with CS. Previous studies on the chelation of Zn ions with CS focused on its applications in adsorption (Nieto, Peniche-Covas, & Zhou, 1999; Mcafee, Gould, Nadeau, & Costa, 2001), metal ions separation or waste water treatment (Denkewicz, Senderov, & Grenier, 2000; Navarro & Tatsumi, 2001; Gyliene, Rekertas, & Salkauskas, 2002). Zn ions possess nutritional features important to human health and health care. Recent years, CS–Zn complex attracted great interests for its potential use as medicament or nutriment (James et al., 2003; Paik, 2001; Yonekura & Suzuki, 2003; Kathryn, 2003). It is well known that both CS and Zn have the properties of disinfection and bactericide (Jeon & Kim, 2000; Takai et al., 2002). After CS binds to Zn ions through nitrogen, oxygen or a combination of them, the bindings are likely to leave some potential donor atoms free and these free donor atoms enhance the biological activity. So it stands a good chance that CS–Zn complexes exhibit enhanced ability of antimicrobial, which will be very favorable to CS–Zn complexes' applications in medical industry and food industry. But up to the present, there are scarcely reports on the antimicrobial activities of CS metal complexes, although the study is necessary and significant.

The present objective is to investigate the antimicrobial activities of CS–Zn complexes and to explore preliminarily the structure–activity correlation. In this paper, five CS–Zn complexes with different Zn content were prepared, and their compositions and structures were analyzed through several physical methods including: elemental analysis, AAS, FT-IR and XRD. The complexes' antimicrobial activities against four gram-positive bacteria, five gram-negative bacteria and two fungi were studied systematically.

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2. Experimental

2.1. Materials

CS from the shrimp shell was obtained from Yuhuan Ocean Biochemical Co. (Zhejiang, China), the degree of deacetylation was 83% and average molecular weight was 788 kDa. Beef extract and peptone were purchased from Shanghai Chemical Agent Co. (Shanghai, China). The other chemicals used were of analytical grade.

The microorganisms tested were provided by China Center for Typical Culture Collection, Wuhan University, except *Cand. Albicans* that was original isolated from patient provided by the people's hospital of Hubei province in China.

2.2. Characterizations

IR spectra were recorded with KBr discs in the range of 4000–400 cm^{-1}on Nicolet-170 SX FT-IR spectrophotometer; elemental analysis (C, H, N) were performed on a Carlo–Erba 1106 elemental analyzer. Metal content was measured by a Hitachi 180–80 atomic absorption spectrometry. X-ray diffraction was recorded by a Rigaku Kmax-r AX diffractometer with scanning scope of 5–40° scanning speed of 4°/min, using Cu Kα radiation.

2.3. Preparation of the complexes

CS (0.3 g) was dissolved in 30 ml acetic acid (1%) By stirring, desired amount of $ZnSO_4·7H_2O$ (corresponding to a molar ratio of 4:1, 2:1, 1:1, 0.5:1, 0.25:1 compared with CS residue) was added into the solution. The pH value was increased to 7.0 by adding 0.1 M $NH_3·H_2O$ solution. The mixture was refluxed at 80 °C for 3 h with stirring. After it was cooled to room temperature, the mixture was poured into 200 ml acetone. The resulting white precipitate was obtained by filtration. The product was repeatedly washed with ethanol and finally dried under vacuum to constant weight.

2.4. Evaluation of antimicrobial activity in vitro

The agar plate method was used to determine the minimum inhibition concentration (MIC) of CS–Zn complexes as follows: the complexes and CS solutions were prepared in 0.3% (v/v) hydrochloric acid at a concentration of 1% (w/v), then they were autoclaved at 121 °C for 25 min. Duplicate two-fold serial dilutions of each sample were added to nutrient broth (beef extract 5 g, peptone10 g to 1000 ml distilled water, pH 7.0) for final concentration of 0.1, 0.05, 0.025, 0.0125, 0.00625, and 0.00313%. $ZnSO_4$ samples were prepared and diluted by the same way except for final concentration of 0.00065, 0.00033, 0.00016, and 0.000082%. Hydrochloric acid (0.3%) was used as a control instead of samples. The culture of each bacterium was diluted by sterile distilled water to 10^5–10^6 CFU/ml. A loop of each suspension was inoculated on nutrient medium with sample or control added. After inoculation, the plates were incubated at 37 °C for 72 h, and the colonies were counted and the MIC values were obtained.

The minimum inhibitory concentration (MIC) was considered to be the lowest concentration that completely inhibited against on agar plates comparing with control, disregarding a single colony or a faint haze caused by the inoculum. (Speciale, Musumeci, Blandino, Milazzo, Caccamo, & Nicoletti, 2002)

3. Results and discussion

3.1. Structure deduction and mechanism discussion

The elemental analysis results showed (Table 1) that the calculated chelate ratios of complexes increased with more Zn ions used in the reaction, although not all Zn ions involved in complexation

The X-ray diffraction spectra of CS–Zn(c), CS and $ZnSO_4·7H_2O$ were shown in Fig. 1. The diffraction spectra of $ZnSO_4·7H_2O$ exhibits of two major crystalline peaks at 2θ 21°, 24°,while CS consisted of two major peaks at 2θ 10°, 20°. CS chelated with Zn sulfate shows more numerous and sharper X-ray diffraction bands than untreated CS. The pattern consisted with the study of Muzzarelli, Ferrero, and Pizzoli (1972), revealing the formation of a new crystalline phase.

The FT-IR spectrum of CS–Zn(c) exhibits many alterations from that of CS (Fig. 2). The major differences are: (1) The wide peak at 3436 cm^{-1}, corresponding to

Table 1
Synthesization of the complexes

Complex	Analysis found (Calc.) %				Mixture ratio	Calculate ratio
	Zn(II)	C	N	H		
CS–Zn (a)	11.17(10.92)	9.98(10.08)	2.08(1.96)	3.65(1.54)	1:4	1:1.2
CS–Zn (b)	10.15(10.23)	15.19(15.12)	2.88(2.94)	4.38(2.31)	1:2	1:0.75
CS–Zn (c)	9.86(10.0)	23.15(22.32)	4.77(4.34)	5.18(3.41)	1:1	1:0.5
CS–Zn (d)	4.38(4.42)	28.94(28.8)	5.68(5.6)	5.57(4.4)	1:1/2	1:0.17
CS–Zn (e)	0.91(0.98)	37.86(36.0)	6.73(7.0)	6.93(5.5)	1:1/4	1:0.03

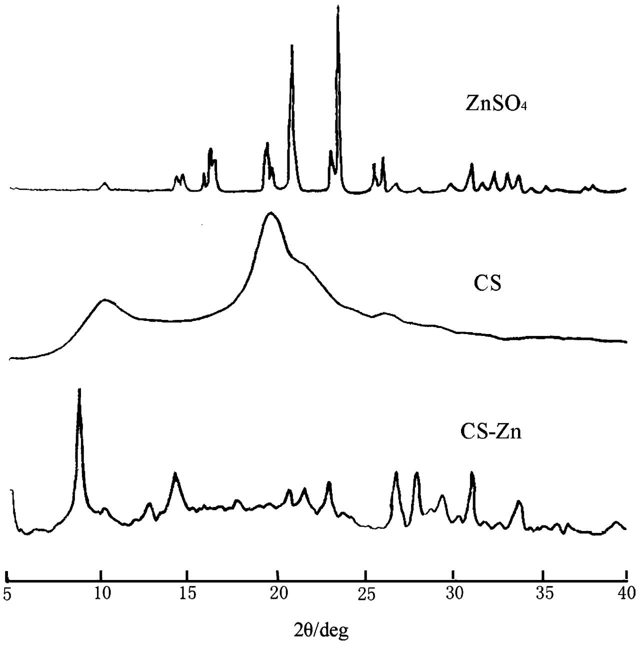

Fig. 1. X-ray diffraction of Chitosan–Zn complex, Chitosan and $ZnSO_4$.

the stretching vibration of $-NH_2$ group and $-OH$ group, shifted to lower frequency (3402 cm^{-1}), with enhanced Zn^{2+}content in the complexes more pronounced shift could be observed (Fig. 3). The absorb band at 1617 cm^{-1} assigned to the bending vibration of $-NH_2$ group (Qin, Xiao, Du, Shi, & Cheng, 2002) shifted to higher wavenumber (1628 cm^{-1}). It indicated that the $-NH_2$ group and $-OH$ group were involved in complexation (Tang, et al., 2001; Hon David & Tang, 2000). (2) The band (1089.6 cm^{-1}) assigned to the second $-OH$ group showed a significant shift to lower wavenumber, which was enhanced with increasing Zn content. It suggested that the second $-OH$ group got involved in complexation (Wang, Zhou, & Yu, 2000).

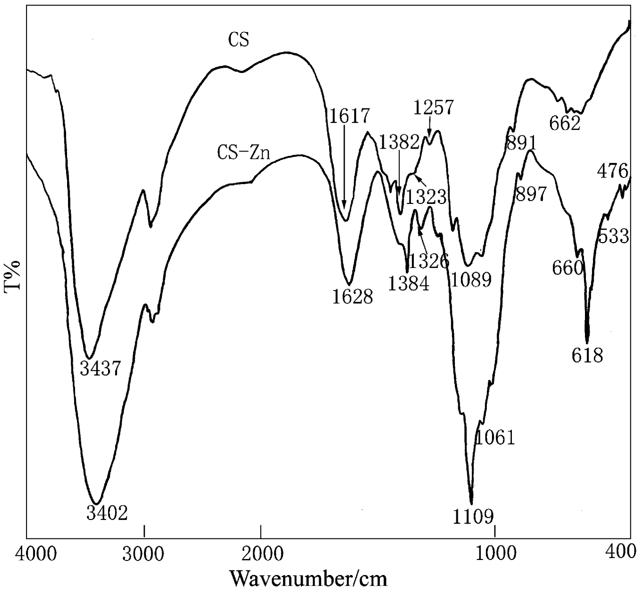

Fig. 2. FT-IR spectra of Chitosan and Chitosan–Zn complexes.

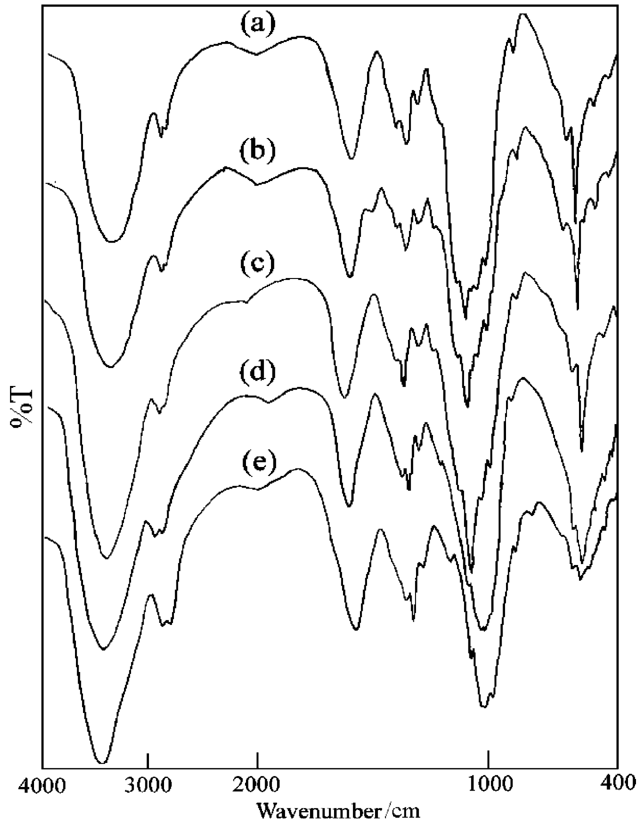

Fig. 3. FT-IR spectra of Chitosan–Zn complexes with different chelate ratio. a, CS–Zn(a); b, CS–Zn(b); c, CS–Zn(c); d, CS–Zn(d); e, CS–Zn(e).

Several new absorb peaks are also observed in the spectra of CS–Zn complexes. The bands near 1109 and 618 cm^{-1} which were due to the stretching vibration of S–O bond (Wang, Zhao, Gao, & Yu, 1999) became weaker with decreasing chelate ratios, revealing decreasing content of SO_4^{2-} in the complexes with decreasing chelate ratios. The phenomena consisted with the result of elemental analysis. New bands were also observed in the range of 533–534 cm^{-1} and 472–476 cm^{-1}, which were assigned to stretching vibration of N–Zn and O–Zn (Tang, et al., 2001).

As a kind of d^{10} ions, Zn(II) usually adopts a tetracoordinate mood with ligands. Different models had been proposed to elucidate the structure of CS and metal ions (Nieto, Peniche-Covas, & Del Bosque, 1992; Piron & Domard, 1998). Those models could be classified into two groups: pendant pattern, in which metal ions were bound to one amino group of CS; and bridge pattern, in which metal ions bond to two or more amino groups and hydroxyl groups of one or more CS chains as a bridge. In this study, the CS–Zn(II) complexes with different chelate ratios were found to chelate different amount of Zn ions, and exhibit diverse properties. The molecule structure of CS–Zn(II) complexes with different chelate ratios showed in Fig. 4.

Fig. 4(1) corresponds to complexes with molar ratio of Zn and CS larger than 1:1, while Fig. 4(2) and (3)

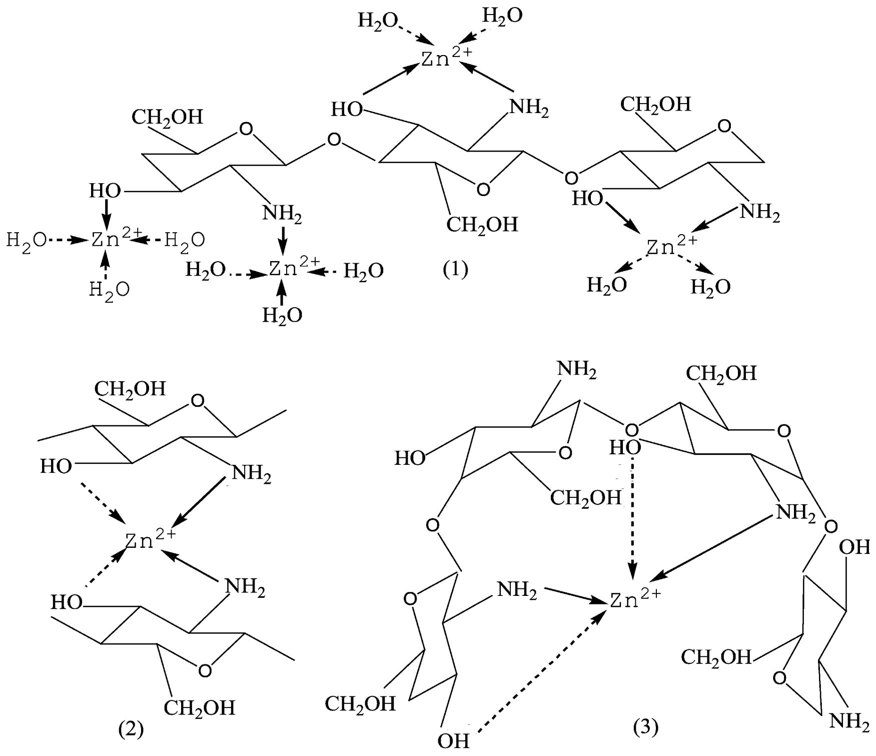

Fig. 4. The reasonable structure of Chitosan–Zn complexes.

correspond to complexes with molar ratios equal to and fewer than 1:1, respectively.

It was also found that the pH value of the system decreased with the process of complexation and decreased more quickly with more Zn content in the complexes. Similar phenomena had also been found in the study of Rhazi, Desbrieres, Tolaimate, Rinaudo, Vottero, & Alagui (2001), when CS interacting with copper ions. The complexes formation could be described based on Lewis acid-base theory: Zn^{2+}ions acting as the acid are the acceptor of a pair of electrons given by CS acting as the base. The reaction was described as follows:

$$\text{Chit-NH}_2 + H_3O^+ \rightarrow \text{Chit-NH}_3^+ + H_2O$$

$$\text{Chit-NH}_3^+ + Zn^{2+} + H_2O \rightarrow (\text{Chit-Zn})^{2+} + H_3O^+$$

3.2. Antimicrobial activities

The antimicrobial activities of CS–Zn(c), CS and $ZnSO_4 \cdot 7H_2O$ are shown in Table 2.

Table 2
The antibacterial activity of chitosan, $ZnSO_4$ and CS–Zn(c)

Microorganisms	Chitosan (CS%,w/v)	Chitosan–Zn complex		$ZnSO_4 \cdot 7H_2O$ (Zn^{2+}%,w/v)
		(CS–Zn%, w/v)	(Zn^{2+}%,w/v)	
Gram-negative bacterial				
Escherichia Coli	0.025	0.00313	0.00022	0.0018
Pseudomonas aeruginosa	0.0125	0.00625	0.00044	0.0036
Proteus mirabilis	0.025	0.00625	0.00044	0.0036
Salmonella enteritidis	0.05	0.00625	0.00044	0.0036
Enterobacter aerogenes	0.05	0.00625	0.00044	0.0036
Gram-positive bacterial				
Staphylococcus aureus	0.05	0.00625	0.00044	0.0018
Corynebacterium	0.025	0.00313	0.00022	0.0018
Staphylococcus epidermidis	0.025	0.0125	0.00044	0.0072
Enterococcus faecalis	0.05	0.0125	0.00088	0.0072
Fungal				
Cand Albicans	0.1	0.1	0.00176	>0.0072
Candida Parapsilosis	0.1	0.05	0.00176	>0.0072

Table 3
The antibacterial activity of Chitosan–Zn complexes with different chelate ratios

Complexes	CS–Zn(a)	CS–Zn(b)	CS–Zn(c)	CS–Zn(d)	CS–Zn(e)
Gram-negative bacteria L					
Escherichia Coli	<0.00313	0.00313	0.00313	0.0125	0.0125
Pseudomonas aeruginosa	0.00625	0.00625	0.00625	0.0125	0.0125
Proteus mirabilis	0.00625	0.00625	0.0125	0.0125	0.0125
Salmonella enteritidis	0.00625	0.00625	0.0125	0.0125	0.025
Enterobacter aerogenes	0.00625	0.00625	0.0125	0.025	0.025
Gram-positive bacterial					
Staphylococcus aureus	0.00625	0.0125	0.0125	0.025	0.05
Corynebacterium sp.	0.00313	0.00313	0.00625	0.00625	0.00625
Staphylococcus epidermidis	0.00625	0.00625	0.0125	0.025	0.025
Enterococcus faecalis	0.0125	0.025	0.025	0.05	0.05
Fungal					
Cand Albicans	0.05	0.05	0.1	0.1	0.05
Candida Parapsilosis	0.05	0.05	0.05	0.1	0.1

It was found that CS–Zn complex showed effectively wide spectrum antimicrobial activities against all of the microorganisms used in the test, although differences existed among different kinds of microorganisms. Generally, the complexes had better antibacterial activity than antifungal activity. There were no obvious differences observed between the complexes' antibacterial activities against gram-positive bacteria with gram-negative bacteria. Among all the bacteria tested, *E. Coli* and *Corynebacterium* were most visibly inhibited by CS–Zn(c), both of which had a MIC value of 0.00031%.

Compared with CS and $ZnSO_4$, CS–Zn(c) had much better antimicrobial activities, whose MIC values were 2–8 times lower than those of CS and 4–16 times lower than those of $ZnSO_4$. It was noticed that the complex was 8-fold more active than CS against three bacteria: *E. Coli*, *S. aureus* and *Corynebacterium*, which were most obviously inhibited by $ZnSO_4$. The phenomena indicated that CS–Zn complex not only remained even enhanced the activities of CS, but also maintained the inhibitory activities of $ZnSO_4$ against some microorganisms.

As a kind of macromolecule polymer, CS seems to be unable to pass the outer membrane of bacteria, since this membrane functions as an efficient outer permeability barrier against macromolecules (Helander, Nurmiaho-Lassila, Ahvenainen, Rhoades, & Roller, 2001). Therefore, direct access to the intracellular parts of the cells by CS is unlikely. The antibacterial mechanisms of CS suggested to be: the positive charge of the amino group at C-2 resulted in a polycationic structure which can be expected to interacted with the predominantly anionic components (lipopolysaccharides, proteins) of the microorganisms' surface (Helander et al., 2001). The interaction resulted in great alteration of the structure of outer membrane (Helander, Latva-Kala, & Lounatmaa, 1998), which caused release of major proportion of proteinaceous material from the cells (Vaara & Vaara, 1983). When chelated with Zn ions, the positive charge on the amino group of CS was strengthened. As a result, the complex was easier to interact with anionic components of cell surface, and exhibited higher inhibitory activities. More work is needed to confirm this hypothesis.

The antimicrobial activities of the five CS–Zn complexes were compared in Table 3.

It can be observed that the antimicrobial activities of CS–Zn complexes enhanced with increasing chelate ratios. The complex with a chelate ratio of 4:1 was 4-fold more active than the complex with a chelate ratio of 0.25:1. As to most microorganisms tested, ideal-inhibiting effects could be obtained when the chelate ratios of complexes were above 1:1. So proper antimicrobial activity of CS–Zn complex could be obtained by controlling the amount of Zn salt in the preparation.

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