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Preparation of high antimicrobial activity thiourea chitosan–Ag^+ complex

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Abstract

Thiourea chitosan was prepared by the reaction of chitosan with ammonium thiocyanate in ethanol. Thiourea chitosan was characterized by FT-IR, ^{13}C NMR, and elemental analysis; XPS confirmed that in thiourea chitosan–Ag^+ complex, S atoms coordinated to silver ions and were the major electron donors; O atoms also coordinated to silver ions and they were another electron donors next to S atoms. N atoms did not take part in coordination. Thiourea chitosan–Ag^+ complex overcomes the instability of Ag^+. Antimicrobial activities of the complex was evaluated against six species of bacteria and molds. The complex showed a wide spectrum of antimicrobial activities, whose MIC values against bacteria were 20 times lower than those of chitosan, 100 times lower than those of sodium diacetate and 200 times lower than those of sodium benzoate, respectively; the complex has a better antibacterial activity than antifungal activity.

Keywords: Chitosan; Thiourea chitosan; Antimicrobial activity; Silver iron; Complex

1. Introduction

Chitosan is a natural nontoxic biopolymer derived by the deacetylation of chitin. Chitosan and its derivatives have attracted considerable interest due to their antimicrobial and antifungal activities (Jung et al., 1999). The antimicrobial activity of chitosan depend on several factors such as the kind of chitosan (deacetylation degree, molecular weight) used, the pH of the medium, the temperature, etc. The mechanism of the antimicrobial activity has not been fully elucidated yet, but several hypotheses have been postulated. The most feasible hypothesis is a change in cell permeability due to interactions between the polycationic chitosan and the electronegative charges on the cell surfaces. This interaction leads to the leakage of intracellular electrolytes and proteinaceous constituents (Chen, Liau, & Tsai, 1998; Feng et al., 2000; Papineau, Hoover, Knorr, & Farkas, 1991; Sudarshan, Hoover, & Knorr, 1992; Young & Kohle, 1982). Other mechanisms are the interaction of diffused hydrolysis products with microbial DNA, which leads to the inhibition of the mRNA and protein synthesis (Hadwiger et al., 1986) and the chelation of metals, spore elements and essential nutrients (Cuero, Osuji, & Washington, 1991).

Silver or silver ions have long been known to have powerful antibacterial activity. Silver is widely applied in some medical fields for its high antimicrobial activity and low concentration (Kang, Jung, & Jeong, 2000). The complex of chitosan with Ag^+ exhibits antibacterial activity (Zhan, Xiong, Liu, & Xie, 2002). However, Ag^+ including the adsorptive Ag(I) by chitosan is instable, and can be easily reduced as Ag, which limits its medical application.

According to hard and soft acid theory, thiourea compounds are very efficient at chelating noble metals and widely used for extracting noble metals including silver, gold, etc. (Chanda & Rempel, 1990). In order to introduce thiourea group into chitosan main chain, thiourea grafting chitosan using glutaraldehyde

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as cross-linking agent was prepared (Guibal, Vincent, & Navarro Mendoza, 2000a; Guibal et al., 2001). However, the obtained thiourea grafting chitosan can not dissolve into any solvent, which restricts its use as antimicrobial. A kind of soluble chitosan modified by thiourea is desired.

In this paper, thiourea chitosan (TU-CTS) was prepared by the reaction of citosan with ammonium thiocyanate, which was easy to be dissolved into 1% acetic acid solution. Thiourea chitosan and Ag^+ formed a stable thiourea chitosan—Ag^+(TU-CTS-Ag^+) complex, which remarkably enhanced the antimicrobial activity of chitosan as well as the stability of Ag^+.

2. Experimental

2.1. Materials

Chitosan with deacetylation degree of 90% was purchased from Yuhuan Organisms Co. Ltd. (Zhejiang Province, China). Beef extract, potato starch, glucose and peptone were purchased from Shanghai Chemical Agent Co. (Shanghai, China).

The microorganisms tested were provided by Xiangtan University in China.

The other reagents were of analytical grade and used without further purification.

2.2. Characterizations of thiourea chitosan and thiourea chitosan–Ag^+

^{13}C NMR spectra were performed on a Bruker (AVACE) AV-500 spectrometer in D_2O/HCl and D_2O/CF_3COOD, respectively; IR spectra were recorded on Nicolet-170 SX FT-IR spectrophotometer in KBr discs; S% was measured in a SC-132 sulfur meter (LECO), while C%, N% and H% were measured by Elemental Analyzer-MOD 1106 (Carlo Erba Strumentazione); Ag^+ content was measured by a Hitachi 180-80 atomic absorption spectrometry. X-ray photoelectron and X-ray excited Auger spectra were obtained with a Physical Electronics PHI 5700 spectrometer with using non-monochromated Mg Kα radiation ($h\nu = 1253.6$ eV) and operating at 15 kV and 20 mA; the vacuum in the analysis chamber was better than 10^{-9} mbar. Binding energies were corrected using the binding energy values for C 1 s of adventitious carbon fixed at 284.8 eV.

2.3. Preparation of thiourea chitosan and thiourea chitosan–Ag^+

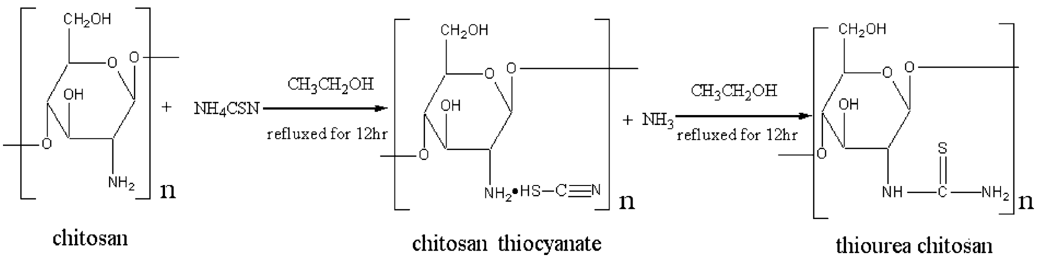
A mixture of 16.1 g (0.1 mol) chitosan powder, 15.2 g (0.2 mol) ammonium thiocyanate and 150 ml ethanol in a three-neck flask with amagenetic stirring was refluxed for 12 h. After cooling down to room temperature, the precipitate was collected by filtration and repeatedly washed with ethanol, then was dissolved in 500 ml of 1% (v/v) acetic acid solution. By adding 10% (w/v) NaOH solution into the solution and filtrating, the precipitate was collected and successfully washed with water and finally dried to give 16.5 g of thiourea chitosan.

0.3 g of thiourea chitosan was dissolved in 30 ml of acetic acid (1%, v/v), followed by addition of 25 ml of $AgNO_3$ solution (0.01 g $AgNO_3$) into the solution by stirring. After the mixture was stirred for 3 h at room temperature, the precipitate obtained by adding 200 ml acetone into the mixture was collected by filtration, successively washed with 95% ethanol, and dried to give thiourea chitosan–Ag^+ complex. The sorption amount of Ag^+ in thiourea chitosan–Ag^+ complex was 1.44 mg/g as determined by atomic absorption spectrometry.

2.4. Evaluation of antimicrobial activity in vitro

The agar plate method was used to determine the minimum inhibition concentration (MIC) of chitosan, sodium diacetate, sodium benzoate and thiourea chitosan–Ag^+ complexe. The 1% (w/v) solutions of chitosan, sodium diacetate, sodium benzoate and thiourea chitosan–Ag^+ were prepared in 1% (v/v) acetic acid. 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 the final concentration of 0.1, 0.05, 0.025, 0.0025, 0.00125, and 0.00025%; 0.1%(v/v) of acetic acid was used as a control instead of samples. Then they were autoclaved at 121 °C for 25 min. 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 the sample or the 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 MIC was considered to be the lowest concentration that completely inhibits against bacteria comparing with the control, disregarding a single colony or a faint haze caused by the inoculum (Speciale, et al., 2002).

Following the procedure described above, slight modifications were made in order to obtain the MIC values of chitosan, sodium diacetate, sodium benzoate and thiourea chitosan–Ag^+ against molds. Potato starch glucose media (potato starch 10 g, glucose 20, agar 20 g and pH value in nature) was used for culturing molds. The culture of each mold spores was diluted by sterile distilled water to 10^5 CFU/ml. A loop of each suspension was inoculated on nutrient medium with the sample or the control added. The inoculated plates were incubated at 30 °C for 10 days, and the colonies were counted and the MIC values were obtained.



Scheme 1. Synthesis of thiourea chitosan.

Table 1
Elemental analysis results of CTS and TU-CTS

Sample	Mole ration of glucosamine unit to ammonium thiocyanate	Elemental analysis (%)			
		S	C	N	H
CTS		0	38.70	8.51	6.53
TSC-CTS I	1:1	0.4	38.07	8.46	6.45
TSC-CTS II	1:1.5	0.5	37.81	8.35	6.38
TSC-CTS III	1:2	0.5	37.75	8.27	6.35
TSC-CTS IV	1:2.5	0.6	37.52	7.85	6.28

3. Results and discussion

3.1. Characterization of thiourea chitosan and thiourea chitosan–Ag^+

A traditional method of introducing thiourea group into chitosan is carried out using glutaraldehyde as cross-linking reagent (Guibal et al., 2000a; Guibal et al., 2001). The obtained product is an insoluble solid and not used for bacteriostasis. Here we used a different approach to introduce thiourea group into chitosan. It is well known that when temperature is above 70 °C, part of ammonium thiocyanate converts into its isomeric compound, thiourea. Our strategy is that ammonium thiocyanate and chitosan are together heated to form chitosan thiocyanate and ammonia; similarly, chitosan thiocyanate is heated to convert into thiourea chitosan. The preparation of thiourea chitosan is illustrated in Scheme 1.

As can be seen from Table 1, with increasing the mole ratio of glucosamine unit to ammonium thiocyanate, sulfur content in thiourea chitosan slightly increased. The sulfur content in thiourea chitosan is less than 0.6%. It indicates that only a small amount of chitosan thiocyanate is converted into thiourea chitosan and the yield is low. Although chitosan thiocyanate is similar to ammonium thiocyanate, the reaction activity of chitosan thiocyanate is lower than ammonium thiocyanate upon heating. Further work is needed to investigate for this difference. However, a small quantity of thiourea chitosan could chelate with a few of silver ions which is and enough to remarkably enhance the antimicrobial activity of chitosan.

Structure changes of thiourea chitosan and thiourea chitosan–Ag^+ were confirmed by FT-IR spectra (Fig. 1). The FT-IR spectra of thiourea chitosan shows new peaks at 1535.1 cm^{-1}, which is assigned to the characteristic absorbance peak of thiourea groups (Padhye, 1985; Rao & Kasturi, 1964); In addition, the signal from amide I at 1646.7.9 cm^{-1} (ν(C=O)) and 1600.2 cm^{-1} (δ(–NH_2)) (Fernandez Cervera et al., 2004) disappeared and was replaced by a new peak at 1631.5 cm^{-1} (δ(–NH_2)) and its intensity became weak as result of the amino group of chitosan being substituted. These results suggest thiourea group are part of chitosan.

Comparing with thiourea chitosan, in the FT-IR spectra of thiourea chitosan–Ag^+, a new peak at 825.4 cm^{-1} appeared, which was assigned to the stretching vibration of S–Ag^+ (Lobana, Bhargava, Sharma, & Kumar, 2003).

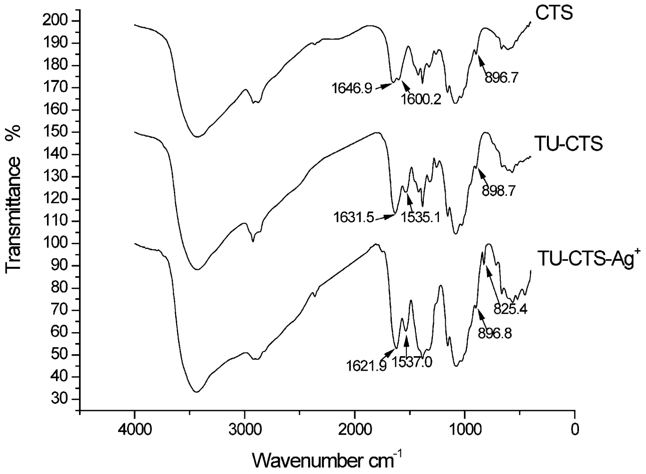


Fig. 1. FT-IR spectra of chitosan, thiourea chitosan and thiourea chitosan–Ag^+.

Fig. 2(a) and (b) show the ^{13}C NMR spectra of chitosan and thiourea chitosan in D_2O/CF_3COOD and D_2O/HCl, respectively. It can be seen that the signals of ^{13}C NMR spectra in Fig. 2(a) are stronger and clearer than in Fig. 2(b), since chitosan and thiourea chitosan have better solubility in D_2O/CF_3COOD than in D_2O/HCl.

Whether in D_2O/CF_3COOD or D_2O/HCl, the ^{13}C-NMR chemical shifts for chitosan at 58.3(C2), 62.5(C6),

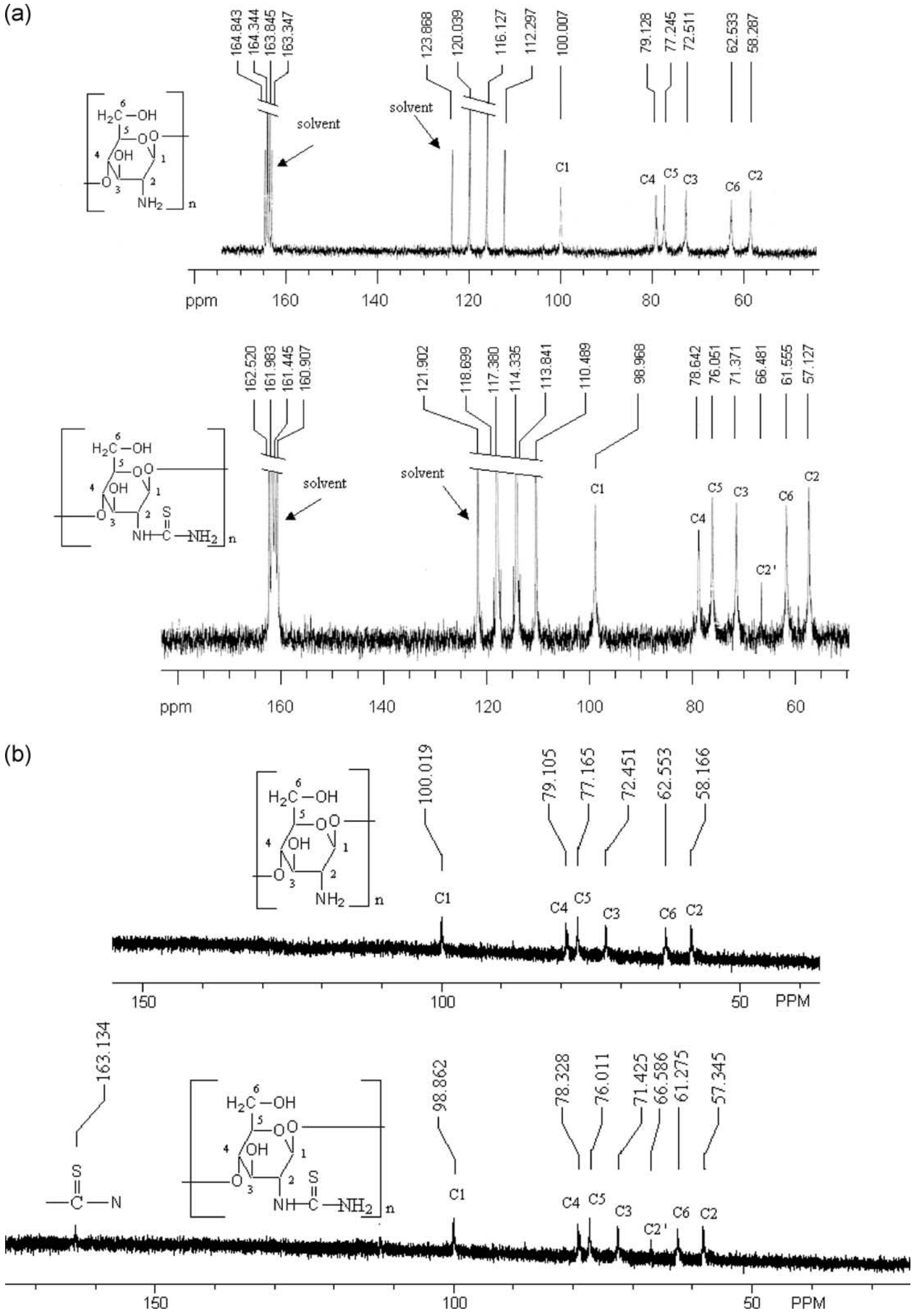


Fig. 2. (a) ^{13}C NMR spectra of chitosan and thiourea chitosan in D_2O/CF_3COOD. (b) ^{13}C NMR spectra of chitosan and thiourea chitosan in D_2O/HCl.

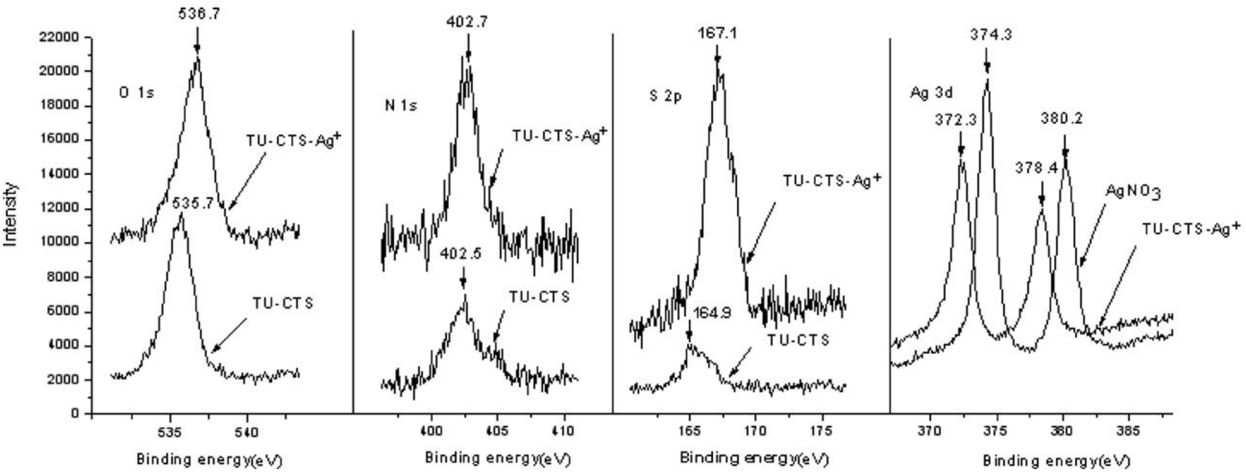


Fig. 3. Ag 3d, S 2p, O 1s and N 1s XPS spectra.

72.5(C3), 77.2(C5), 79.1(C4) and 100.0(C1) (ppm) were detected (Dung, Milas, Rinaudo, & Desbriers, 1994; Rinaudo, Desbrieres, le Dung, Thuy Binh, & Dong, 2001). In contrast to chitosan, the signals for thiourea chitosan upshifted and appeared at 57.1(C2), 61.6(C6), 71.4(C3), 76.1(C5), 78.6(C4) and 98.9(C1) (ppm). It was noticeable that the weaker signals for substituted C2 remarkably downshifted and appeared at 66.5 ppm (C2′). Hence, it could be concluded that a few of C2 amino groups were substituted by thiourea groups because the stronger signals for unsubstituted C2 at 57.1 ppm still were observed.

Comparing Fig. 2(a) with Fig. 2(b), the weaker signals at 163.3 ppm in Fig. 2(b) was assigned to the carbons of thiourea groups (Klimova, Klimova, Martinez Garcia, Mendez Stivalet, & Ruıiz Ramirez, 2001), which were superimposed by the stronger signals for C=O in CF_3COOD and could not be identified in Fig. 2(a). The ^{13}C-NMR spectrum suggests C2 amino group of chitosan was substituted by thiourea group.

The evidences of elemental analysis, FTIR, and ^{13}C-NMR obviously support the amino groups of chitosan were partly converted into thiourea groups.

As well known, XPS is a very useful tool for detecting the interaction between builders in complex. Fig. 3 shows the Ag 3d, S 2p, O 1s and N 1s XPS spectra.

Ag3d electron spectra for $AgNO_3$ presented two signal peaks with the binding energy (BE) of 374.3 and 380.2 eV. After Ag^+ chelating with thiourea chitosan, the peaks significantly shifted to 372.3 and 378.4 eV, respectively; this result indicates that the electron density at the Ag atoms is higher than that of the free ligand; the S 2p electron spectra of the complex show the BE value is significantly shifted to 167.1 from 164.9 eV, indicating that the electron density at the S atoms is greatly less than that of the free ligand. This implies the coordination of S atoms to silver ions and S atoms are the major electron donors; similarly, the electron density at the O atoms is lower than that of the free ligand, which is confirmed by the BE shifting from 535.7 eV to 536.7 eV in the O 1s electron spectra of the complex; O atoms are another electron donors next to S atoms. The BE value of N1s is close to that of the free ligand, indicating no coordination of N atom. The thiourea chitosan chelating with Ag^+ could be described based on hard–soft-acid–base (HSAB) theory: Ag^+ acting as a soft acid is the acceptor of electrons given by S and O atoms in thiourea chitosan acting as a soft base.

3.2. Antimicrobial activities

The antimicrobial activities of chitosan, thiourea chitosan–Ag^+ complex and sodium diacetate sodium benzoate are shown in Table 2.

Table 2
Antibacterial activity of CTS, TU-CTS-Ag^+, sodium diacetate and sodium benzoate

Antibacterial Microorganisms	CTS(w/v, %)	TU-CTS-Ag^+		Sodium diacetate (w/v, %)	Sodium benzoate (w/v,%)
		(TU-CTS-Ag^+%, w/v)	(Ag^+%, w/v)		
Staphylococcus aureus	0.05	0.0025	0.000012	0.25	0.5
Eschercihia Coli	0.05	0.0025	0.000012	0.25	0.5
Bacillus subtilis	0.05	0.0025	0.000012	0.25	0.5
Aspergillus flavus	0.5	0.05	0.00024	0.5	0.5
Mucor kacilliformis	0.5	0.05	0.00024	0.5	0.5
Paccilomgces uarioti	0.5	0.05	0.00024	0.5	0.5

It can be seen that thiourea chitosan–Ag^+ complex shows a wide spectrum of antimicrobial activities against all of the microorganisms tested. It is also noticed that the complex has good antibacterial activity and antifungal activity. Generally, the complexes has better antibacterial activity than antifungal activity.

Although only a few silver ions are loaded on thiourea chitosan, compared with chitosan, sodium diacetate and sodium benzoate, thiourea chitosan–Ag^+ has much better antimicrobial activities, whose MIC values against bacteria were 20 times lower than chitosan, 100 times lower than sodium diacetate and 200 times lower than sodium benzoate, respectively. The chelation Ag^+ significantly enhanced the antimicrobial activities of chitosan. Generally, the inhibitory effect of silver on bacteria can be explained by the reaction of Ag^+ with proteins by combing the –SH groups of enzymes, which leads to the inactivation of the proteins. Another inhibition mechanism of silver ions on microorganism is believed that silver ions affect DNA molecules by losing their replication abilities. (Feng, et al., 2000).

4. Conclusion

Thiourea chitosan was prepared by the reaction of chitosan with ammonium thiocyanate in ethanol. Thiourea chitosan was characterized by FT-IR, ^{13}C NMR, and elemental analysis; XPS confirmed that in thiourea chitosan–Ag^+ complex, S atoms coordinated to silver ions and were the major electron donors; O atoms were another electron donors next to S atoms. Thiourea chitosan–Ag^+ complex improved the instability of Ag^+.

In vitro antimicrobial activities of the complex were evaluated against six species of bacteria and molds. The complex showed a wide spectrum of effective antimicrobial activities. Its MIC values much lower than those of chitosan, sodium diacetate and sodium benzoate. The complex has better antibacterial activity than antifungal activity.

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