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Carbohydrate Polymers 55 (2004) 291–297
Carbohydrate Polymers
www.elsevier.com/locate/carbpol

Structural characterization and antimicrobial activity of chitosan/betaine derivative complex

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Received 8 June 2003; revised 18 July 2003; accepted 15 October 2003

Abstract

Complex of chitosan acetate (CSAC) with C12–C18 alkyl amido prophyl dimethylamine betaine (AAPDB) was prepared and characterized by Fourier transform-infrared (FT-IR), thermal analysis (TG, DTG and DSC), X-ray diffractogram. Results showed that complex formed mainly by the electrostatic interaction between NH_3^+ of CSAC and COO^- of AAPDB. Minimum inhibitory concentrations (MICs) were evaluated against *E. coli* ATCC 25922, *Pseudomonas aeruginosa* ATCC 27853, *Staphylococcus aureus* ATCC 25923, *Staphylococcus epidermidis* and *Candida albicans*. Results showed that CS/AAPDB solution could or even more strongly inhibit growth of all the tested microorganisms, whereas CSAC could not inhibit growth of *C. albicans* and AAPDB could not inhibit growth of *S. epidermidis*, *E. coli*, *P. aeruginosa*. Relative inhibition times (RITs) of CS/AAPDB solutions with different concentration and ratio of CSAC and AAPDB were also investigated against the five microorganisms. Results showed that CS/AAPDB solutions with $C_{CSAC} + C_{AAPDB}$ of 0.05% + 0.015%, 0.05% + 0.0075%, and 0.025% + 0.0075% showed higher antimicrobial activity. The relationship between complex formation and antimicrobial activity was discussed.

Keywords: Chitosan; Betaine derivative; Complex; Antimicrobial activity

1. Introduction

Chitosan, the deacylated derivative of chitin, is a nontoxic copolymer consisting of β-(1,4)-2-acetamido-2-deoxy-D-glucose and β-(1,4)-2-amino -2-deoxy-D-glucose units. It has attracted considerable interests due to their biological activities such as antimicrobial (Choi et al., 2001; Jeon, Park, & Kim, 2001; No, Park, Lee, & Meyers, 2002; Roller & Covill, 1999), antitumor (Suzuki et al., 1986), and immune enhancing effects (Sugano, Yoshida, Ilashimoto, Enomoto, & Ilirano, 1992), etc.

As a new natural antimicrobial agent, the antibacterial and antifungal activities of chitosan (Shahidi, Arachchi, & Jeon, 1999) and its derivatives such as *N*-sulfonated and *N*-sulfobenzoyl chitosan (Chen, Liau, & Tsai, 1998), carboxymethylchitosan (Chen, Du, & Liu, 2000) and quaternary ammonium salt of chitosan (Jia, Shen, & Xu, 2001) were reported widely. But few reports on antimicrobial potency of chitosan in combination with other interfering substances were found until now, except for some food products (Hoades & Roller, 2000). Surfactants can be used in many products such as detergents, cleaning products, some agricultural agents, etc. There is no report about application of chitosan as an antimicrobial agent in these products. To explore the application of chitosan in these products, we studied the antimicrobial activity of chitosan in a betaine derivative due to the good compatibility.

Betaines, inner salts and zwitterionic compounds, containing a carboxylate groups and a quaternary ammonium group, play an important role in many living systems as methyl transfer agents, osmoprotectors and osmoregulators. Complexes of these bases can be important as model systems because of role of the hydrogen bonds in many biologically important reactions (Godzisz, Ilczyszyn, & Ilczyszyn, 2002). These facts explain a wide interest in studies on the betaine and its complexes. Betaines containing a hydrophobic chain of 8–20 carbon atoms are surface active agents often added to other surfactants to largely improve their performance, mildness, foaming, viscosity of other properties (Domingo, 1996).

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doi:10.1016/j.carbpol.2003.10.001

The purpose of this study was to evaluate the antimicrobial activity of chitosan in betaine derivative through comparing with chitosan and betaine derivative alone, and attempt to investigate the interaction of chitosan acetate with betaine derivative and the relationship between the interaction and antimicrobial activity, so as to explain the mechanisms on different antimicrobial activity of them with different molecular properties.

2. Experimental

2.1. Materials

Chitosan (CS) from a shrimp shell was purchased from Yuhuan Ocean Biochemical Co. (Zhejiang, China), molecular weight was 781 kDa and deacetylation degree was 85.5%. The molecular weight was measured by a gel permeation chromatography, and the deacetylation degree was determined by elemental analysis. Amphoteric C12–C18 alkyl amido prophyl dimethylamine betaine 30% aqueous solution (AAPDB) was obtained from Huntsman Surface Sciences UK Limited, West Midlands B68 ONN. Other chemicals are reagent grade.

2.2. Preparation of CS/AAPDB Complex

CS was dissolved into 1% (w/v) acetate buffer (pH 4.8), chitosan acetate (CSAC) was obtained and the final concentration of CSAC was adjusted to 1% (w/v). AAPDB was diluted to 1% aqueous solution (pH 6). CS solution was dropped into same volume of AAPDB solution. The resultant solutions were carefully stirred for at least 2 h, and then stored for at least 1 day at room temperature allowing equilibration. Complexes with different concentration and ratio of CS and APPDB were prepared by changing the initial concentration of CS and APPDB solutions. The final solutions were transparent. These solutions were directly used for assays of antimicrobial activity. Films of the CS/AAPDB were cast from the above solution after dialyzing against distilled water for more than 3 days on a glass plate at room temperature for a few days and finally dried to constant mass under vacuum at 50 °C for several days. Pure CSAC and APPDB were also prepared under the same conditions.

2.3. Characterizations

IR spectra were taken on KBr pellets on a 170SX Fourier transform-infrared (FT-IR) spectrophotometer (America Nicolet company) by the method of transmission.

TG, DTG and DSC curves of samples were performed by a Setaram Setsys 16 TG/DAT/DSC (France) under nitrogen atmosphere of 0.15 Mpa and argon atmosphere of 0.10 Mpa from 20 to 600 °C at a heating rate of 10 °C/min.

X-ray diffraction patterns were measured by a D_{max}-ray diffractrometer and used a Cu Kα target at 40 kV and 50 mA.

2.4. Microorganisms and culture conditions

E. coli ATCC 25922, *Pseudomonas aeruginosa* ATCC 27853, *Staphylococcus aureus* ATCC 25923 for antimicrobial assay were provided by Typical Cultural Collection Center in Wuhan University, China. *Staphlococcus epidermidis* and *Candida albicans* were donated by the People's Hospital of HuBei province in china. Bacteria were incubated on nutrient agar (peptone 1%, beef extract 0.5%, NaCl 0.5%, agar 2%, pH 6) at 37 °C for 1 day, while *C. albicans* at 28 °C for 2 days.

2.5. Assays for antimicrobial activity

To prepare microorganism suspension, take one or several colonies of microorganisms in agar plates with sterile tampon into sterile saline (0.9%) solution, then diluted to 10^5–10^6 colony/ml. Sample solutions were autoclaved at 121 °C for 15 min. One millilitre of sample solutions and 9 ml autoclaved nutrient agar were poured into autoclaved petri-dishes, cooled, one ring of microorganism suspension was drawn as line on cooled nutrient agar, then incubated at 37 °C for bacteria, 28 °C for *C. albicans*. Observed and recorded whether colonies were visible with naked eye after regular incubation times. All treatments were triplicate.

Minimum inhibitory concentration (MIC) was tested as follows: sample solutions were two-fold diluted serially, then operated as above. The MIC was defined as the lowest concentration of the tested sample at which the microorganism colonies were not visible with naked eye within 19–38 h. Relative inhibition time (RIT) was indicated by the difference between the time when microorganism colonies were visible in agar plates with tested samples and in controlled plates.

3. Results and discussions

3.1. Complex formation of CSAC with AAPDB

3.1.1. Fourier transform-infrared study

In our experiment, CS/AAPDB was dialyzed against distilled water for more than 3 days, if AAPDB was not formed complex with CSAC, it would be removed out from the system. And then there would be no special absorption band of AAPDB in the IR spectra of CS/AAPDB (dried from solution containing 0.5% CSAC and 0.15% AAPDB). But in Fig. 1, we can see that the absorption band at 2921, 2851 and 719 cm^{-1}, which was caused by C–H (Williams & Fleming, 1995) of alkyl chain in AAPDB, were very obvious. This indicates that AAPDB formed complex with CSAC.

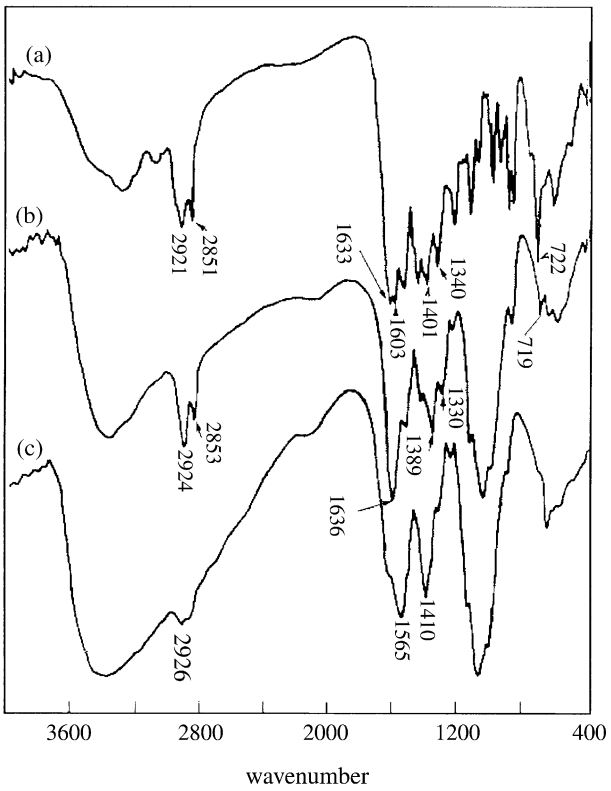

Fig. 1. FT-IR spectra of AAPDB(a), CS/AAPDB (b) and CSAC(c).

As well known, FT-IR is a very useful tool for detecting the interaction in polymer blends, so in this article, FT-IR was employed to examine the possible interactions between the blend components. Fig. 1 showed the FT-IR spectra of CSAC, AAPDB and CS/AAPDB. In these spectra, the weak absorption band at 2800–2000 cm^{-1} was attributed to the NH_3^+ overtones (Williams & Fleming, 1995). Compared with the spectra of CSAC and AAPDB, the band of CS/AAPDB had a relatively stronger absorbent, which indicated that the new hydrogen bond was possibly formed between CSAC and AAPDB. The absorption bands at 1633 cm^{-1} of AAPDB spectrum and 1650 cm^{-1} of CSAC spectrum were assigned to the amide I band (Williams & Fleming, 1995).

For AAPDB, the absorption band at 1603 cm^{-1} was due to the asymmetrical COO^- stretching vibration, and the absorption band at 1401 cm^{-1} was due to symmetrical COO^- stretching vibration (Peniche et al., 1999). In the spectrum of CS/AAPDB, the band at 1603 cm^{-1} shifted to the place at 1636 cm^{-1} and overlapped with the amide 1 band. The band at 1401 cm^{-1} shifted to the place at 1389 cm^{-1}. At the same time, C–N stretching vibration of CSAC at 1334 cm^{-1} shifted to the place at 1330 cm^{-1}. These indicated the electrostatic interaction between COO^- of AAPDB and NH_3^+ of CSAC possibly occurred. In addition, the absorption band of CSAC at 1565 and 1410 cm^{-1} due to the asymmetrical and symmetrical COO^- stretching vibration (Huang, Du, & Yang, 2003; Peniche et al., 1999) disappeared in CS/AAPDB. This indicated that CH_3COO^- was removed from CSAC by dialysis. This result further testified the interaction of NH_3^+ in CSAC with AAPDB. What is more, C–N stretching vibration of AAPDB at 1340 cm^{-1} shifted to the place at 1330 cm^{-1}, which indicated the quaternary ammonium groups were also likely participated interaction.

3.1.2. Thermal analysis

TG (a) and DTG (b) curves of CS/AAPDB, CSAC and AAPDB are shown in Fig. 2. From TG and DTG curves we can see that CSAC degraded in three stages. It was reported that TG curves of CS showed two stages (Qin, Du, & Xiao, 2002; Tirkistani, 1998). The first degradation stage of CS was explained as the loss of water. The second stage was due to the degradation of CS, and the greatest weight loss point was at 298 °C (Qin et al., 2002) or 334 °C (Tirkistani, 1998), with weight loss of 49% (Tirkistani, 1998). In our study, the first and third stages of CSAC are very similar to the two stages of CS reported. The difference between CS and CSAC is that acetic acid was introduced into CSAC. Hence, it is likely that the second stage of CSAC was attributed to the loss of acetic acid.

TG curve of CS/AAPDB was very different from CSAC and AAPDB. It showed two stages. The first water-loss stage

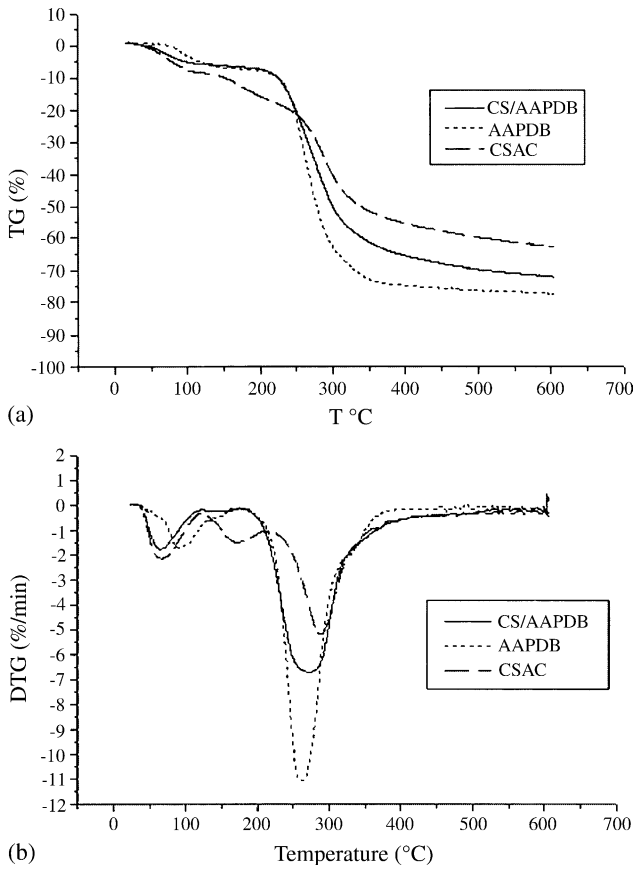

Fig. 2. TG (a), DTG (b) curves of CS/AAPDB, AAPDB and CSAC.

Table 1
The temperature of endothermic and exothermic peaks from DSC curves of CS/AAPDB, CSAC and AAPDB

Samples	Endothermic peaks, temp. (°C)	Exothermic peaks, temp. (°C)
CSAC	103, 171	267
CS/AAPDB	100, 175	206, 254
AAPDB	96, 182	216, 258

started at ~50 °C and reached a maximum at 68 °C, with weight loss of ~6%. T_{max} of first stage was lower than AAPDB (90 °C) and CSAC (70 °C). The second degradation stage of CS/AAPDB started at ~180 °C till 600 °C and reached a maximum at ~270 °C, with weight loss of ~63%. T_{max} was lower than CSAC (290 °C), but higher than AAPDB (260 °C). The weight remains after 600 °C was ~25%, which was also between CSAC (39%) and AAPDB (20%). And interestingly, CS/AAPDB did not show the second stage of CSAC, which was likely due to the loss of acetic acid. Hence, in CS/AAPDB, the acetic acid was likely removed. DTG curves showed these results more clearly. DTG curve of CC/AAPDB showed two obvious peaks. The second peak of CSAC disappeared in curve of CC/AAPDB. This result is in agreement with that from FT-IR spectra.

The endothermic and exothermic peaks from DSC curves are summarized in Table 1. CSAC had two endothermic peaks at 103 and 171 °C and an exothermic peak at 267 °C. It was reported (Ritthedej, Phaechamud, & Koizumi, 2002) that chitosan had an endothermic peak at ~100 °C which was associated with the evaporation of water and an exothermic peak at 306 °C which was corresponding to the decomposition temperature of this polymer. They also reported the endothermic and exothermic peaks from DSC curves of chitosan salts. Results showed that chitosan citrate, formate, glycolate, lactate and malate all had an endothermic peak at 158–198 °C except for chitosan acetate and propionate. In my experiment, CSAC also showed an endothermic peak at 171 °C, which was similar to most of chitosan salts. The different results may be caused by the process of drying, because amide group will be formed due to the moist heat treatment (Ritthidej et al., 2002). Hence, it seems that the endothermic peak at 158–198 °C was associated to the electrostatic interaction of protonated amino group in the chitosan backbone with carboxylate ion in the carboxylic acids. Similarly the endothermic peaks of CS/AAPDB and AAPDB were likely due to the electrostatic interaction of ammonium in CSAC and quaternary ammonium group in AAPDB with $-COO^-$ in AAPDB, respectively. The exothermic peak of CSAC (267 °C) and CS/AAPDB (254 °C) was shifted to lower temperature compared to that of chitosan (306 °C). These results are in good agreement with chitosan salts reported (Ritthidej et al., 2002). And the temperature of exothermic peak of CS/AAPDB was lower than that of CSAC and AAPDB.

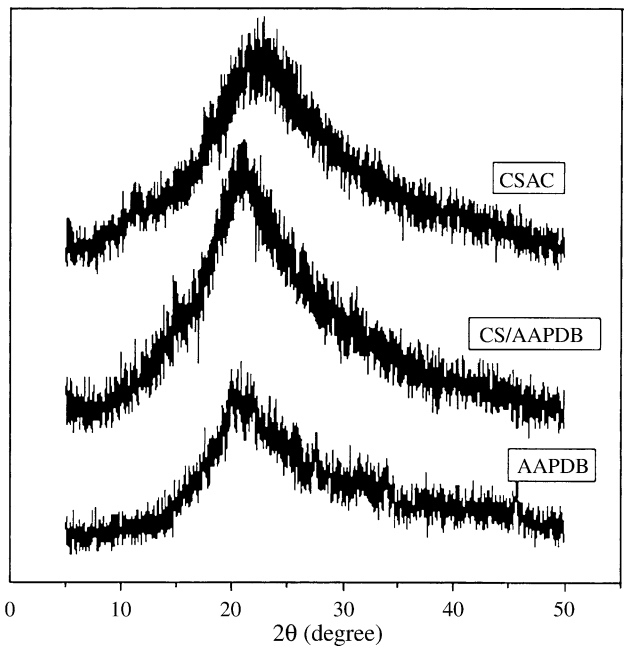

Fig. 3. X-ray diffraction patterns of CSAC, AAPDB and CS/AAPDB.

3.1.3. Powder X-ray diffraction study

The X-ray diffractograms of CS/AAPDB, CSAC and AAPDB are shown in Fig. 3. It could be seen that they all had one major peak, but there were some differences of peak height, width and position between them. CSAC showed a relatively broader peak at 23° 2θ, with height of 225. The peak of CS/AAPDB at about 21° 2θ was much narrower than CSAC. And the peak height of CS/AAPDB (275) was higher than that of AAPDB (140) and CSAC. It is well-known that the width of X-ray diffraction peak is related to the size of crystallite, the broadened peak usually results from small crystallites. Peaks at smaller angle, corresponding to larger spacing, mean the decrease of the crystalline perfection (Gao, Shao, Sun, & Lin, 2000). Hence, CS/AAPDB had larger size but also larger spacing of crystallites than CSAC and higher crystallinity than CSAC and AAPDB.

From above discussion, it can be seen that complex of CSAC with AAPDB was formed, most likely due to the electrostatic interaction between $-NH_3^+$ of CSAC and $-COO^-$ of AAPDB.

3.2. Antimicrobial activity

In Table 2, as to compare with CSAC and AAPDB, the initial concentration of CSAC and AAPDB in CS/AAPDB solution was selected close to the MICs of CSAC and AAPDB. As seen in Table 2, at tested conditions, CS/AAPDB could inhibit the growth of all the five tested microorganisms, whereas CSAC could not inhibit *C. albicans* and AAPDB could not inhibit *S. epidermidis*, *E. coli* and *P. aeruginosa*. Hence, CS/AAPDB showed broader inhibition range. And MICs of CS in CS/AAPDB

Table 2
MICs (%) of CS/AAPDB comparing with CS and AAPDB

Treatments	*S. aureus* G^+ bact.	*S. epidermidis* G^+ bact.	*E. coli* G^- bact.	*P. aeruginosa* G^- bact.	*C. albicans* fungi
Acetate buffer	(0.1)	(0.1)	(0.1)	0.05	(0.1)
CSAC	0.05	0.05	0.025	0.025	(0.1)
AAPDB	0.0075	(0.1)	(0.1)	(0.1)	0.0038
CS/AAPDB	0.05/0.0075	0.05/0.0075	0.0125/0.0019	0.0063/0.0009	0.025/0.0038

Ineffective at the tested concentrations in bracket.

against *S. aureus* and *S. epidermidis* were same as that of CSAC, but that against *E. coli* and *P. aeruginosa* were lower two and four times, respectively. As for *C. albicans*, CS/AAPDB kept the same MIC with AAPDB alone. Hence, antimicrobial activity of CS/AAPDB strengthened comparing with CSAC and AAPDB.

The exact mechanism of the antimicrobial action of chitosan is still unknown, but different mechanisms have been proposed (Shahidi, Arachchi, & Jeon, 1999). As a polymeric macromolecule with molecular weight of 781 kDa, chitosan cannot directly access to the intracellular parts of the cells. A key feature of this chitosan is its positive charge of the amino group at C-2 below its pK_a (pH 6.3). This creates a polycationic structure which can be expected to interact with anionic components of cell surface (Papineau, Hoover, Knorr, & Farkas, 1991; Sudharshan, Hoover, & Knorr, 1992). In addition, chitosan can bind on the cell surface (Helander et al., 2001) to form a film around the cells, so the transport of nutrient into cells is disturbed (Zheng, Zhu, & Sun, 2000). Chelating of metal ions such as Ca^{2+} and K^+ by chitosan (Cuero, Osuji, & Washington, 1991) should also be put into consideration.

As discussed above, the NH_3^+ of CSAC is likely interacted with the COO^- of AAPDB. Hence, the interaction of NH_3^+ in chitosan with anionic components of cell surface should be weakened. But in CS/AAPDB, a quaternary ammonium group and a C_{12}–C_{18} alkyl chain were introduced in. Quaternary ammonium group can also interact with phospholipids of cell membrane (Thomas, Brock, & Michael, 1991), so disrupt microbial membrane, and may also denature proteins (Prescott, Harley, & Klein, 2002). –OH groups in CS/AAPDB could likely chelate metal elements, such as Ca^{2+}, which is important in cell membrane. In addition, the introduction of C_{12}–C_{18} alkyl chain will make CS/AAPDB in more proximity to and dissolve the cell membrane. Hence, CS/AAPDB showed cooperative antimicrobial activity. This can also explain why CS/AAPDB showed stronger antimicrobial activity on gram-negative bacteria whose most outer layer is outer membrane and which have thin cell wall, whereas gram-positive bacteria have thicker cell wall and no outer membrane. As for AAPDB, the negative charge of COO^- affected the interaction of quaternary ammonium group with negatively charged cell surface, so it showed lower antimicrobial activity.

On the other hand, the higher antimicrobial activity of CS/AAPDB than AAPDB may be also due to the high molecular weight. Though they both have cationic groups and alkyl chains, CS/AAPDB with high molecular weight could form a layer of film around cells to affect the absorption of nutrients.

Table 3 showed the RIT of CS/AAPDB with different concentration and ratio of CSAC to AAPDB. It can be seen that when the concentration of CS was kept at 0.05%, and the concentration of AAPDB decreased from 0.015 to 0.001%, all CS/AAPDB mixture showed inhibition effect on the four tested bacteria, only those with AAPDB concentration of lower than 0.0038% showed shorter RIT. But for

Table 3
RITs (h) of CS/AAPDB with different concentration and ratio of CSAC and AAPDB

Sample	Concentraions	*S. aureus* G^+bacteria	*S. epidermidis* G^+ bacteria	*E. coli* G^- bacteria	*P. aeruginosa* G^- bacteria	*C. albicans* fungi
CS/AAPDB	0.05% + 0.015%	>120	>120	>120	>120	>101
	0.05% + 0.0075%	>120	>120	>120	>120	24
	0.05% + 0.0038%	>120	>120	>120	>120	14
	0.05% + 0.0019%	72	>120	72	>120	0
	0.05% + 0.001%	48	>120	48	>120	0
	0.025% + 0.0075%	>120	>120	24	24	>101
	0.0125% + 0.0075%	>120	0	0	0	>101
	0.0063% + 0.0075%	>120	0	0	0	>101
	0.0031% + 0.0075%	24	0	0	0	>101

C. albicans, RIT of CS/AAPDB decreased with decreasing of AAPDB concentration. Theoretically, with the decreasing of AAPDB concentration, complex formation decreased, the antimcirobial activity of CS/AAPDB mixture should decrease just as that of *C. albicans*. But the excess CS could also inhibit growth of bacteria, so CS/AAPDB with lower AAPDB concentrations also showed antibacterial activity. When the concentration of AAPDB was kept at 0.0075%, and the concentration of CSAC decreased from 0.05 to 0.0031%, RIT of CS/AAPDB mixture decreased obviously, some of them lost activity. This is because the complex formation of CS/AAPDB decreased with the decreasing of CSAC concentration, and the excess AAPDB could only inhibit growth of *S. aureus* and *C.albicans*. Hence, ratio of CSAC to AAPDB markedly affected the inhibition range. Sequentially, CS/AAPDB with $C_{CSAC} + C_{AAPDB}$ of 0.05% + 0.015%, 0.05%+ 0.0075%, and 0.025% + 0.0075% showed higher antimicrobial activity against both tested bacteria and fungi.

4. Conclusions

The results of IR spectra, thermal analysis and X-ray diffractograms showed that complex of CS/AAPDB formed and mainly due to the electrostatic interaction between NH_3^+ of CSAC and COO^- of AAPDB. This complex showed stronger antimicrobial activity and broader inhibition range than CSAC and AAPDB alone. And CS/AAPDB with $C_{CSAC} + C_{AAPDB}$ of 0.05% + 0.015%, 0.05% + 0.0075%, and 0.025% + 0.0075% showed higher inhibition effect. Hence, chitosan can be used as antimicrobial agent with AAPDB in many products in which need surfactant.

The possible reasons for the stronger antimicrobial activity of CS/AAPDB are summarized as follows: (i) introduction of quaternary ammonium group from AAPDB, which can disrupt microbial membrane, and may also denature proteins; (ii) introduction of alkyl chain from AAPDB, which can make CS/AAPDB in more proximity to and dissolve the cell membrane; (iii) introduction of a large amount of –OH from CSAC, which may chelate metal elements such as Ca in cell membrane; (iv) properties of higher molecular weight from CSAC, which may make it form a layer of film to affect the absorption of nutrients. As to make clear about the exact mechanisms on antimicrobial activity of them, further researches are being done in our laboratory to determine the interaction between them and cell surface such as cell wall and cell membrane.

Acknowledgements

The work was supported by the National Natural Science Foundation of China (grant No. 29977014).

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