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Physicochemical characterization and antibacterial property of chitosan acetates

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Abstract

In a new approach to the preparation of solid chitosan acetate, the dependence of solubility of chitsoan acetate on the mole ratio of acetic acid to GlcN residues of chitosan was evaluated from turbidity. The structure of the product chitosan acetate was characterized by titration and FT-IR. It was demonstrated that the chitosan acetate with high solubility retained the structure and antibacterial activity of chitosan. The antibacterial activities against *Escherichia coli* (Gram negative) and *Staphylococcus aureus* (Gram positive) have been exhibited by a special kind of agar and further investigated by optical density method. It was found that chitosan acetate at a concentration of 0.1% (m/V) exhibited some antibacterial activity but some of the bacteria did still grow. With a concentration of 0.15% (m/V), there were nearly no bacteria grown. Electron micrographs revealed bacterial action patterns of chitosan acetate towards *E. coli* and *S. aureus*.

Keywords: Chitosan acetate; Antibacterial activity; *Escherichia coli*; *Staphylococcus aureus*

1. Introduction

Chitin is a natural polysaccharide which is usually obtained from shells of crustaceans such as crab, shrimp, and crawfish. Chitosan is a partially *N*-deacetylated derivative of chitin and consists of polymeric (1 → 4)-linked 2-amino-2-deoxy-β-D-glucopyranose units. Due to the unique polycationic nature, chitosan and its derivatives have been proposed for various applications in biomedical, food, agricultural, biotechnological and pharmaceutical products (Devlieghere, Vermeulen, & Debevere, 2004).

Chitosan has a broad spectrum of antimicrobial activities (Chung, Wang, & Chen, 2003), and high bactericidal rates (Helander, Nurmiaho-Lassila, Ahvenainen, Rhoades, & Roller, 2001) and low toxicity toward mammalian cells, making it a potential biocide in food preserving (Roller & Covill, 1999). However, the poor solubility of chitosan at neutral and higher pH limits its applications. Some modification has been attempted, such as quaternary ammonium salts of chitosan, *N*-alkylated chitosan derivatives with monosaccharides, and chitosan-*O*-poly(ethylene glycol) graft copolymers prepared to improve water solubility and bioactivities of chitosan (Gorochovceva & Makuska, 2004; Jia, Shen, & Xu, 2001; Yang, Chou, & Li, 2002). But the traditional method of modifying is to introduce some covalent bond, which is usually complex and sometimes changes some of the properties of chitosan (Huang, Du, Zheng, Liu, & Fan, 2004).

In this paper, a new method of preparing solid chitosan acetate is reported. The chitosan acetate has a structural formula as shown in Fig. 1. Chitosan and acetic acid were linked by electrovalent bond in the chitosan acetate rather than covalent bond, i.e., this is a salt because of the existence of electrovalent bond. Chitosan acetate was soluble in

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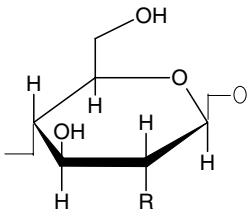

R= $NH_3^+.CH^3COO^-$, NH_2

Fig. 1. Structural formula of chitosan acetate.

water because of the protonation of $–NH_2$. The aqueous solution of chitosan acetate was similar to the one of chitosan dissolved in acetic acid. So chitosan acetate as a salt would still keep the structure of chitosan and antibacterial activity of chitosan. Furthermore, it was more stable than chitosan solution (dissolved in acetic acid) because of its solid state and more convenient in use than chitosan because of its water solubility. The *in vitro* antibacterial activities of chitosan acetate against *Staphylococcus aureus* and *Escherichia coli*, which are two main waterborne food pathogens, were evaluated by an optical density method.

2. Materials and methods

2.1. Materials

Chitosan was made from shrimp shell and obtained from Biochemical Medicine Plant of Qingdao (Qingdao, China). The degree of deacetylation was 89% as determined by titration (Jia & Li, 2001) and the average molecular weight of chitosan was 82 kDa as determined by viscometric method. The other chemicals were of analytical grade and were used without further purification.

2.2. Preparation of chitosan acetate

Chitosan (5.0 g) was suspended in aqueous ethanol 5:95%, v/v (40 ml). The desired different amounts of acetic acid according to different mole ratio of acetic acid to GlcN residues of chitosan (HAc:GlcN, 1:1, 2:1, 3:1, 4:1, 5:1) were added into chitosan suspensions to yield different chitosan acetate samples. After stirring for 2 h, the resulting white precipitate was taken as the product and dried under vacuum to constant weight.

2.3. Characterizations of chitosan acetate

The water solubility of chitosan acetate was evaluated from turbidity measurements based on the method of Kubota, Tatsumoto, Sano, and Toya (2000) after some modification. Dried chitosan acetate was dissolved in distilled water at was 1.0%, w/v. The relationship between the mol ratio of acetic acid to GlcN residues of chitosan and the solubility of chitosan acetate was estimated, different chitosan acetate samples having been prepared in Section 2.2 according to different mol ratios of acetic acid to GlcN residues of chitosan. Studies were performed in triplicate and average values with standard deviation errors were reported.

For the stability assessment, dried chitosan acetate was stored at 25 °C for different durations of days at room temperature and the stability of chitosan acetate was assessed by viscosimetry. Dried chitosan acetate (0.4 g) was dissolved with stirring for 1 h in distilled water (20 ml) and the viscosity of the solution was measured by using a rotational viscometer. The pH and optical density at 420 nm turbidimetry measurements of the salt solution were based on dried chitosan acetate (0.2 g) dissolved with stirring for 1 h in distilled water (20 ml).

The content of acetic acid in the chitosan acetate was determined by titration achieved with the method of Lee, Ha, and Park (1995) after slight modification. Dried chitosan acetate (0.2 g) was dissolved with stirring for 4 h in distilled water (20 ml) and was titrated with a solution of 0.1 M NaOH by using a pH meter for $–NH^{3+}$ in the chitosan acetate. Chitosan (0.2 g) dissolved in 0.1 M HCl (20 ml) was tested with the same method.

The IR spectrum of the chitosan acetate was recorded on a Fourier Transform Infrared Spectrometer at room temperature, based on the method of Shigemasa, Matsuura, Sashiwa, and Saimoto (1996). The test pellet was formed from 2 mg chitosan acetate and 100 mg of KBr.

2.4. Evaluation of antibacterial activity in vitro

Staphylococcus aureus and *E. coli* were inoculated in nutrient broth (beef extract 5 g, peptone 10 g in 1000 ml distilled water, pH 7.0) (Wang, Du, & Liu, 2004). The inoculation was conducted at 37 °C for 36 h with shaking at 110 rpm. The bacterial suspension obtained was then diluted with the same peptone solution as required.

The effect of chitosan acetate on growth of *S. aureus* and *E. coli* was determined using agar plates. Nutrient broth (10 ml), which had been autoclaved at 121 °C for 20 min was poured into the plate followed by inclining it until there was nearly no broth at one end of the plate. When the agar has solidified, nutrient broth (10 ml) with a chitosan acetate concentration of 0.80% (w/v) was poured onto the plate with the plate laying flat. So the plate was prepared for the final concentration of chitosan acetate, 0.00–0.40% (w/v), from one end to the other. Hundred microliters of the bacteria which was diluted to 10^2 times was pour-plated onto the plate. The plates were prepared in triplicate and incubated at 37 °C for 48 h.

The antibacterial activities against *S. aureus* and *E. coli* of chitosan acetate were carefully measured optically at 610 nm. The bacterial suspension was then diluted to 10^4 times and 1 ml of each bacterium suspension was inoculated into 50 ml the same peptone solution with different amounts of chitosan acetate dissolved in it. The inoculation was conducted at 37 °C for 48 h with and the ODs of the cells were measured spectrophotometrically at 610 nm.

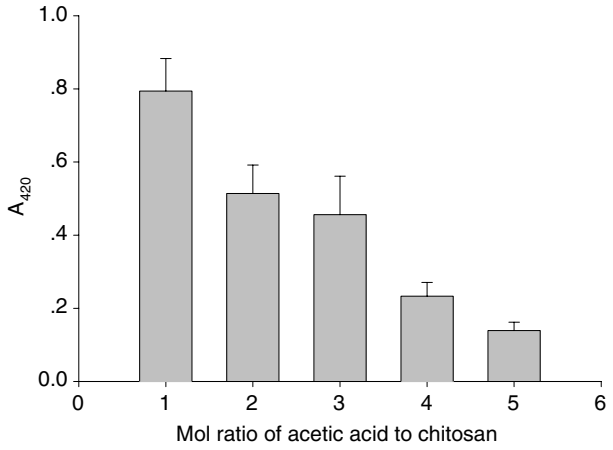

Fig. 2. Effect of the relation between the mole ratios of acetic acid to chitosan on the water solubility of chitosan acetate — A_{420} turbidimetric analysis of the solution of chitosan acetate. The error bars indicate the standard deviation.

Studies were performed in triplicate and average values with standard deviation errors were reported.

2.5. Transmission electron microscopy

Escherichia coli and *S. aureus* cells grown for 48 h were incubated with chitosan acetate as described above. After incubation, the cells were washed twice with PBS and fixed in a fixative 2%, w/v, glutaraldehyde, 2%, w/v, paraformaldehyde, and 0.5%, w/v, $CaCl_2$ in 0.1 M cacodylate buffer. Samples were postfixed in 1.33% (w/v) osmium tetroxide, dehydrated with the aid of aqueous ethanol solutions or increasing ethanol content (from 70 to 100%, v/v) and then infiltrated with a 1:1 (v/v) mixture of propylene oxide. The samples were then embedded in Spurr low viscosity embedding medium. Thin sections of the specimens were viewed under a transmission electron microscope.

3. Results and discussion

3.1. Characterizations of chitosan acetate

Fig. 2 showed the effects of the mole ratios of acetic acid to GlcN residues of chitosan on the water solubility of chitosan acetate. The water solubility obviously depended on the mole ratio of acetic acid to GlcN residues of chitosan. The absorbance decreased when the mole ratio was changed from 1.0 to 5.0, indicating increasing of the solubility. The ionic intensity might be a cause of this phenomenon. When the ionic intensity reached a certain degree, chitosan acetate with high solubility was obtained.

The stability of chitosan acetate for 90 days is shown in Table 1. The pH, viscosity and water solubility of the chitosan acetate did not change obviously during the storage. It indicates that the solid chitosan acetate is stable. Under the same storage condition, the chitosan acetate acid solution was not stable in viscosity, and the viscosity of chitosan solution decreased within a few days (data not shown).

Table 1
Stability of chitosan acetate after storage

Time (days)	pH	Viscosity (mPa S)	Water solubility (turbidity at 420 nm)
1	5.03	0.343	0.037
7	5.01	0.372	0.043
30	5.09	0.473	0.026
60	5.01	0.409	0.074
90	5.07	0.357	0.039

Potentiometric titration of chitosan acetate gave a curve (Fig. 3) having one inflexion point, while the reference (chitosan in 0.1 M HCl) had two inflexion points. The first and second inflexion points of chitosan were the equivalence points of the titration of excess HCl and the titration of protonated chitosan, respectively. The titration curve (Fig. 3) of chitosan acetate had only one inflexion point, the reason for which might be that only a small excess of acetic acid was in the chitosan acetate and acetic acid could not be titrated like HCl since it is a weak acid. It was also calculated that the content of acetic acid in 0.2% (w/v) chitosan acetate was about 0.05% (w/v), which is near to and even lower than the concentration of acetic acid usually used in the essays of antibacterial activity of chitosan as well as some of the chitosan derivatives (Chen, Wu, & Zeng, 2005).

In the FT-IR spectra of chitosan and chitosan acetate (Fig. 4), there were shifts in the amide I and II bands at 1653 and 1590 cm^{-1}, respectively (Osman & Arof, 2003). For the chitosan acetate, the amide II band had disappeared, indicating the occurrence of the formation of $-NH_3^+ \cdot CH_3COO^-$ on the amino groups of chitosan molecules. The appearance of a band at 1558 cm^{-1} for the formation of $-NH_3^+ \cdot CH_3COO^-$ on the amino groups of chitosan acetate molecules is commensurate with this.

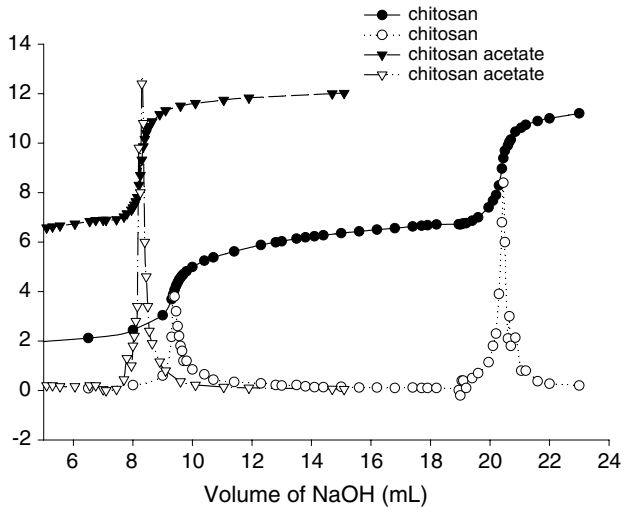

Fig. 3. Titration curve of chitosan acetate with NaOH. Chitosan was dissolved in 0.1 M HCl and chitosan acetate in distilled water. The closed symbols (▼ and ●) represent the relation between pH and the volume of NaOH and the open symbols (▽ and ○) represent the ratio of pH to the volume of NaOH.

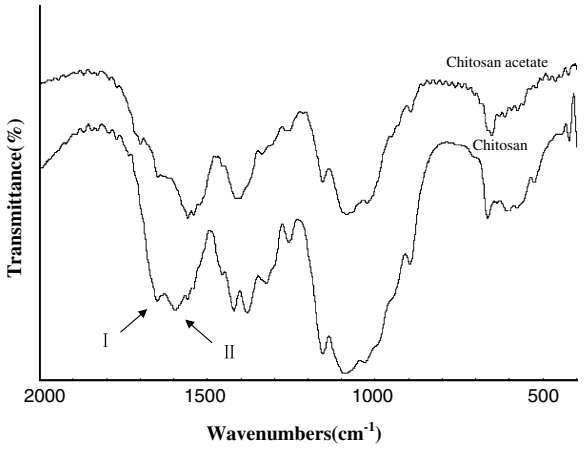

Fig. 4. FT-IR spectra of chitosan and chitosan acetate.

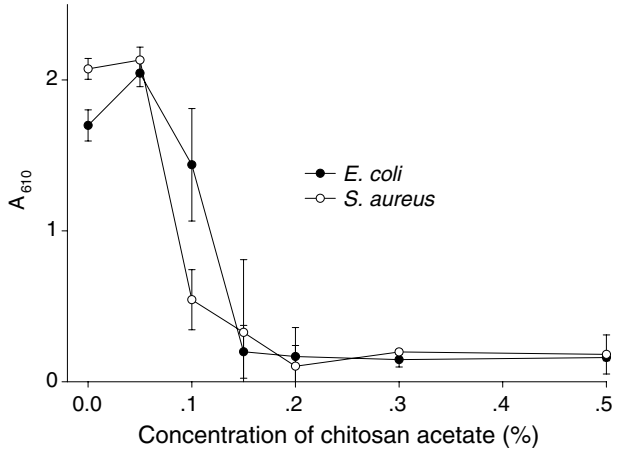

Fig. 5. Growth inhibition of *S. aureus* and *E. coli* in the presence of chitosan acetate as measured turbidimetrically at 610 nm. The error bars indicate the standard deviation.

3.2. Evaluation of antibacterial activity in vitro

In the agar plates observations, it could be seen that with increase of the concentration of chitosan acetate, the antibacterial activity of chitosan acetate against *E. coli* and *S. aureus* increased. The concentration of chitosan acetate in the plates from the left to the right was increased from 0.00% (w/v) to 0.40% (w/v). And *S.aureus* and *E.coli* were inoculated on the plates, the amount of colonies reflecting the viable population decreased from left to right. It inhibited that the antibacterial activity increased with the increasing of the concentration of chitosan acetate.

The inhibitory activities of chitosan acetate were assessed at the following concentrations: 0.00, 0.05, 0.10, 0.15, 0.20, 0.30, 0.50 w/v (Fig. 5), using optical density to measure growth. In the beginning, the growth of *S. aureus* was higher than *E. coli* and it might depend on the initial inoculation level or the difference between the two microbial species. At the concentration of chitosan acetate of 0.05% (w/v), both of the bacteria grew more than the ones with no chitosan acetate. It has been reported that the hydrolytic susceptibility of chitosan to a wide range of enzymes derived from various bacterial, fungal, mammalian, and plant sources is significant (Hirano, Tsuchida, & Nagao, 1998; Pantaleone, Yalpani, & Scollar, 1992). So there was a possibility that when the concentration of chitosan acetate was insufficiently high to inhibit growth of *S. aureus* and *E. coli*, chitosan could be being hydrolyzed by some enzymes in the bacteria and used as a kind of energy. Chitosan acetate at a concentration of 0.10% (w/v) exhibited some antibacterial activity, but some of the bacteria did still grow at a concentration of 0.15% (w/v). It should be mentioned that acetic acid also had antibacterial activity suggesting a cooperative effect of chitosan and acetic acid in the relative studies. However, the content of acetic acid in chitosan acetate was very low (0.05%, v/v) as mentioned above according to the titration curve and it was much lower than had been used previously (Liu et al., in press). So it was mainly the effect of chitosan that was being measured in the study of the antibacterial activity of chitosan acetate.

Chitosan acetate generally showed stronger antibacterial effects on Gram-positive bacteria (*S. aureus*) than on Gram-negative bacteria (*E. coli*) in the presence of 0.10–0.20% (w/v) aqueous chitosan acetate. This conclusion agreed with other reports (No, Park, Lee, & Meyers, 2002). It was probably due to the differences in cell wall structure. The peptidoglycan layer of the cell wall of *S. aureus* is composed of networks with plenty of pores, so chitosan could enter the cell without difficulty. But the cell wall of *E. coli* is made up of a thin membrane of peptidoglycan and an outer membrane composed of lipopolysaccharide, lipoprotein, and phospholipids. Because of this structure of the outer membrane, chitosan could not enter the cells and so the antibacterial activity on *E. coli* was lower than on *S. aureus*. Therefore, chitosan acetate had different effects on *E. coli* and *S. aureus*, similarly to the effects of chitosan (Xie, Xu, & Liu, 2002).

Many other works (Jeon & Kim, 2000; Liu et al., in press) have been done with chitosan (not acetate) dissolved in acid solution, which are in agreement with this work which demonstrates that chitosan acetate can keep its original chitosan structure and hence antibacterial activity.

Chitosan acetate at a concentration of 0.20% (w/v) completely prevented growth of both organisms for the duration of the experiments (48 h). But with the increase of concentration of chitosan acetate, some precipitation occurred and influenced the measurement of optical for concentrations of 0.30 and 0.50% (w/v). Therefore, no conclusion can be drawn for these concentrations. There were two possibilities: (1) chitosan acetate might interact with and flocculate some contents in the broth by the method of ionic interaction (Shimojih, Fukushima, & Kurita, 1998) and (2) the bacteria adhere to each other through the chitosan adsorbed on their surfaces. Strand, Nordengen, and Ostgaard (2002) reported that *E. coli* was flocculated by chitosan at certain concentrations.

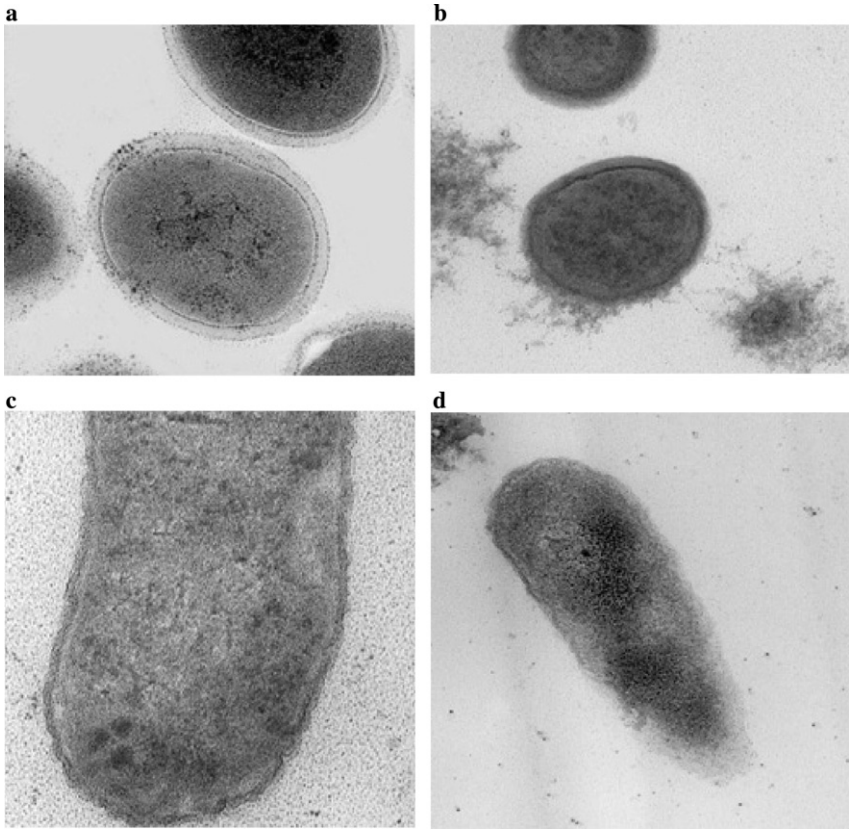

Fig. 6. Electron micrographs of *S. aureus* treated without (a) or with (b) chitosan acetate and *E. coli* treated without (c) or with (d) chitosan acetate. (a–d) ×30,000, (b) ×20,000.

3.3. Transmission electron microscopy (TEM)

The electron micrographs of *E. coli* and *S. aureus* showed the disruption of cell membrane after a period of exposure to chitosan acetate (Fig. 6) and there was some chitosan adhering to the surface of bacteria. A similar finding was reported by Liu, Du, Wang, and Sun (2004). It could be conferred that chitosan acetate interacts and forms polyelectrolyte complexes with acidic polymers produced at the bacterial cell surfaces. The inhibitory effects of chitosan acetate on the growth of *E. coli* and *S. aureus* were different due to the differences in cell wall structure. These results show that the site of chitosan acetate action on cells of the bacteria was on the membrane and that the killing of the organism might be the result of membrane disruption or penetration.

4. Conclusions

Solid chitosan acetate was prepared by the reaction of chitosan with acetic acid. The chitosan acetate was soluble in water. It was confirmed that the chitosan acetate kept the original structure of chitosan and had good water solubility as well as stability under formal storage. *In vitro* antibacterial activities of the complex were evaluated against *S. aureus* and *E. coli*. The solid chitosan acetate salts showed similar antibacterial activities to chitosan. They could inhibit the growth of both bacteria completely at a concentration of 0.20% and chitosan acetate showed stronger bactericidal effects on Gram-positive bacteria (*S. aureus*) than on Ggram-negative bacteria (*E. coli*). TEM of chitosan acetate-exposed cultures of *E. coli* and *S. aureus* revealed signs of membrane disruption and the adherence of chitosan to the outer membrane of the bacteria. In short, chitosan acetate retained the original chitosan structure and antibacterial activity of chitosan and could be more easily used as a preservative in foods that chitosan *per se*.

Acknowledgements

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