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Chitosan kills *Escherichia coli* through damage to be of cell membrane mechanism

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ABSTRACT

This paper aims to identify the interaction of chitosan (CTS) with bacterial membrane and delineate the inhibition mechanism of CTS towards Gram-negative bacteria. The inhibition activity of CTS against *Escherichia coli (E. coli)* was evaluated by measuring $O.D_{610nm}$. CTS have increased the permeabilities of the outer membrane (OM) and inner membrane (IM) of *E. coli*, and ultimately disrupted the cell membrane with the release of cellular contents. Morphologies of bacteria treated with CTS were observed by Transmission electron microscopy (TEM), which indicated that it was the primary inhibition action for CTS to damage the bacterial cell membranes. The interaction between CTS and a model phospholipids membrane was studied, and the precipitates formed with CTS and phosphatidylcholine (PC) suggested the damage of bacterial cell membranes was likely to be caused by the electrostatic interaction between $-NH_3^+$ groups of CTS and carbonyl and phosphoryl groups of the phospholipid components of the cell membrane.

1. Introduction

CTS [poly(β-(1 → 4)-2-amino-2-deoxy-D-glucose)] is the *N*-deacetylated derivative of chitin, found naturally in the exoskeletons of insects, shells of crustaceans, and in fungal walls. CTS has a wide range of applications (No & Meyers, 1995; Sudarshan, Hoover, & Knorr, 1992) and can inhibit the growth of bacteria and fungi. Moreover, CTS has several advantages over other types of disinfectant, that is, it possesses a higher antibacterial activity, a broader spectra of activity, a higher killing rate, and lower toxicity toward mammalian cells (Franklin & Snow, 1981; Takemono, Sunamoto, & Akasi, 1989). However, the actual mechanism for the inhibition activity of CTS is not yet fully understood but has been suggested to involve cell lysis, breakdown of the cytoplasmic membrane barrier, and the chelation of trace metal cations that could be necessary for the microorganism's growth by the CTS (Chung et al., 2004; Helander, Nurmiaho-Lassila, Ahvenainen, Rhoades, & Roller, 2001; Vishu Kumar, Varadaraj, Lalitha, & Tharanathan, 2004). Young et al. suggested that the surface of bacterial cell is composed of negatively charged components, such as lipopolysaccharide and teichoic acids, and an electrostatic interaction between CTS and the negatively charged bacterial cell surface plays an important role in their antibacterial activity (Young, Kohle, & Kauss, 1982). Helander et al. reported that the binding of CTS to the OM of Gram-negative bacteria forming a vesicular structure, causes disruption and extensive alteration to the OM surface and result in the loss of its barrier properties. It is also possible that, due to its ability to interact with lipopolysaccharides (LPS), CTS caused changes in the bacterial membrane permeability, thus enhancing the non-protein nitrogenous substance uptake and released the LPS from the cell surface (Helander et al., 2001). Tharanathan and Kittur showed that CTS binds to DNA thus inhibiting its transcription (Tharanathan & Kittur, 2003). Tokura et al. demonstrated that the stacking of CTS molecules over the microbial cell surface, thus blocking the transport of nutrients (Tokura, Ueno, & Miyazaki, 1997). Papineu et al. observed that the inhibition mechanisms of CTS may lie in damage to the membrane and leakage of cellular materials, ultimately leading to cell death (Papineau, Hoover, Knorr, & Farkas, 1991).

Generally speaking, the mechanism of inhibition activity differs with the molecular weight (Mw) (Hirano & Nagao, 1989; Jeon, Park, & Kim, 2001; No, Park, Lee, & Meyers, 2002), degree of deacetylation (D.D) (Tsai, Su, Chen, & Pan, 2002) and the type of bacterium (No et al., 2002). The bacterial surface is anionic, and offers potential sites for electrostatic binding of protonated CTS. Thus, CTS can interact with lipoid protein complexes in the microbial cell membrane, and alter the permeability of microbial cell membrane (Nikaido, 1996). As for Gram-positive bacteria, CTS could adsorb on the surface of the cell, and thus make the negative charges of cell membrane and cell wall distribute unbalanced. Meanwhile, the intracellular components such as water, proteins tend to leach out, resulting in bacterial lysis and death (Yang et al., 2000; Ye, He, Gao, & Li, 2004). CTS with high Mw can not pass through the

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microbial membrane and hence stack on the cell surface, which blocks nutrient transport to the microbial cell membrane, resulting in cell lysis (Cuero, Osuji, & Washington, 1991; Tokura et al., 1997).While CTS with low Mw can pass through the spongy cell wall. Owing to their small size and being water soluble, they can traverse the microbial membrane (especially for Gram-negative bacteria) (Coma, Deschamps, & Martial-Gros, 2003; Vishu Kumar, Varadaraj, Gowda, & Tharanathan, 2005) and regulate DNA transcription (Tharanathan & Kittur, 2003). In addition, CTS can also bind to trace metals, distorting the membrane structure or its water binding capacity may deprive water availability for microbes causing cell death (Cuero et al., 1991).

It was supposed that CTS firstly interact with the cell membrane of bacteria, which was frequently taken for the antibacterial target. So far, relatively little experimental evidence of the interaction of CTS with the cell membrane is available. To investigate the mechanism of action and the effect of CTS Mw on its inhibition activity, we have firstly studied the inhibition activity of three kinds of CTS to the growth of *Escherichia coli*, then studied the interaction of CTS with the *E. coli* cell membranes. To elucidate detailed inhibition modes of action against bacteria, the integrity of the *E. coli* cell membranes, OM and IM permeability assays were investigated. Information on the morphologies of bacteria treated with CTS has also been shown. In addition, to further investigate the effect of CTS on the cell membrane, the interaction of CTS with phospholipid membranes was studied using FT-IR, thermogravimetry (TG) and differential thermogravimetry analyser (DTA).

2. Materials and methods

2.1. Materials

CTS, from a shrimp shell with Mw of 1000, 50 and 3 kDa and deacetylation degree of 95%, were purchased from Yuhuan Ocean Biochemical (Zhejiang, China). Egg phosphatidylcholine (PC) was obtained from Sigma–Aldrich China (Shanghai, China). γ-GT and LDH assay kit (Merck, Darmstadt, Germany) were used for the determination of γ-GT and LDH activity, respectively. UV–vis spectrophotometer (Rayleigh, UV-9200, Beijing, China). CTS solution was prepared in 1% (v/v) acetic acid. *E. coli* (ATCC 35218) was provided by the Chinese medicine hospital of Tianshui, Gansu, China. Other chemicals were of analytical grade and were used without further purification.

2.2. Inhibition assessment

The inhibition activity of CTS solution against *E. coli* was evaluated by $O.D_{610nm}$, as described by Nan Liu (Liu et al., 2006). *E. coli* was cultivated in a nutrient agar (peptone 10 g, beef extract 3 g, NaCl 3 g, distilled water 1000 ml, pH 7.4) and incubated overnight at 37 °C. A representative colony was picked off and placed in sterile physiological saline. The final cell suspension was adjusted to an absorbance of 0.7 at 630 nm. One milliliter of the cultured bacteria solution was added to 4 ml of 0.5% (w/v) CTS and 15 ml of broth medium autoclaved at 121 °C for 20 min. Then, the plates were incubated overnight on the rotary shaker (128 rpm) at 37 °C. The inhibitory effects were estimated periodically by measuring $O.D_{610nm}$ values. For a blank control group, 4 ml of 1% (v/v) acetate acid in place of the CTS solution was added to the above broth culture. Each batch experiments were carried out three times.

For determination of the minimum inhibitory concentration (MIC) of CTS, CTS was added to nutrient agar for final concentrations of 0.25%, 0.20%, 0.15%, 0.10%, 0.05% and 0.025% (w/v). The MIC was defined as the lowest concentration of CTS required to completely inhibit bacteria growth after incubation at 37 °C for 24 h (Farag, Daw, Hewedi, & El-Baroty, 1989).

2.3. Integrity of cell membrane

E. coli cell membrane integrity was examined by determination of the release of material absorption value at 260 nm (Chen & Cooper, 2002) and 280 nm. The cultured bacteria was harvested, washed twice and resuspended in sterile physiological saline. The final cell suspension was adjusted to an absorbance at 630 nm ($O.D_{630nm}$) of 0.6 and $O.D_{420nm}$ of 0.8 to measure the $O.D_{260nm}$ and $O.D_{280nm}$, respectively. The CTS solutions (50 kDa) of 0.1% (w/v) was mixed with *E. coli* suspension to the ratio of 1:1 (v/v), and the release over time of materials absorbing at 260 and 280 nm were recorded with a UV–vis spectrophotometer.

2.4. OM permeability assays

The OM permeability of *E. coli* was determined by measuring the absorption of CTS to bacteria described by Cheng, Song, Wang, & Gao (1997). The cultured *E. coli* was harvested, washed and resuspended in Na_2HPO_4 (10 mmol/L)-$MgCl_2$ (5 mmol/L, pH 7.0) solution. The final cell suspension was adjusted to $O.D_{570nm}$ of 0.8. A 0.1% (w/v) of CTS was mixed with the cell suspension to the ratio of 1:1 (v/v), and centrifuged at 11,000*g* for 10 min, then the supernatant was extracted with a sterile needle tube, the residual cell suspension were incubated on rotary shaker (128 rpm/min) at 37 °C for 30 min to determine the absorption at 422 nm. The sequence was as follows, 1 ml of 3.33×10–4 mol/L Alizarin red, 1 ml of NaAc-HAc buffer (pH 5.0) and 1 ml of the supernatant was ordinally added into cuvette, the absorbance at 422 nm determinated (Gao, Jiao, Ding, & Chen, 2003).

2.5. TEM

E. coli was prepared for electron microscopy as previously described (Helander et al., 2001). One milliliter of *E. coli* culture with the concentration 10^8/ml was added into CTS solution, to give a final concentration of CTS of 0.1% (w/v). After incubation on a rotory shaker (128 rpm) at 37 °C for 24 h, the suspension was centrifuged. The cells were washed twice with 5 mmol sodium phosphate buffer solution (pH 7.2, PBS) and fixed with 3.0% (v/v) glutaraldehyde in 5 mmol/L PBS. The samples were postfixed with 1% (w/v) OsO_4 in 5 mmol/L PBS for 1 h at room temperature, and washed three times with the same buffer, dehydrated separately at 4 °C for 10 min in a graded series of ethanol solutions(70%, 80%, 90%, 100%, v/v), then embedded in Epon 812 a low-viscosity embedding medium. Thin sections of the specimens were cut with a diamond knife on an Ultracut Ultramicrotome (Super Nova; Reichert-Jung Optische Werke, Wien, Austria) and the sections were double-stained with saturated uranyl acetate and lead citrate. The grids were examined with a JEM-1230 transmission electron microscope (Hitachi, Tokyo, Japan) at an operating voltage of 75 kV.

2.6. Determination of endoenzyme activity in the bacterial suspension

An *E. coli* suspension with a concentration 10^8/ml was divided into several cuvettes, CTS with Mw 50 kDa solution was added to the above suspension which made the final concentration of CTS to 0.1% (w/v). The control was the bacterial suspension but with equal volumes of sterile water instead of CTS solution. Then the samples were centrifuged and filtered, the filtrate was analysed by automatic biochemical analyser (JEM-7170, Hitachi, Tokyo, Japan) to determine the endoenzyme activities.

2.7. Interaction of CTS with phospholipid membrane

Phosphatidylcholine (PC) liposomes were prepared in PBS solution as previously described (Ibrahim, Sugimoto, & Aoki, 2000; Yang, Cui, & Yang, 2002). Dry lipids PC was dissolved in a chloroform–methanol mixture to the ratio of 98:2 (v/v) in a small glass vessel. The solvents were removed under a stream of nitrogen by rotary evaporation to form a thin film on the wall of a glass vessel. Any residual solvents were eliminated by storing the resultant PC in a vacuum oven at 40 °C for 48 h. The lipid was resuspended in PBS buffer (pH 6.0) to the final concentration of 0.5% (w/v), and then the turbid milky mixture was sonicated placed in an ice-water bath until the solution became transparent.

A 0.5% (w/v) of CTS solution and PC liposome were mixed thoroughly with constant vortexing with the ratio of 1:1 (v/v) as previously described (Liu, Du, Wang, & Su, 2004). After stired for 1 h, the mixture was centrifuged at 10000 rpm/min at −2 °C for 10 min. The precipitate was formed in films and dried in a vacuum oven at 50 °C for 48 h, and finely divided before characterization. IR spectra (4000–400 cm^{-1}) of CTS, PC and CTS/PC were taken on KBr pellets with a Spectrum One FT-IR (Perkin Elmer). TG-DTA was carried out using a Pyris Diamond TG/DTA with an aluminum crucible in an atmosphere of air; the experiments were performed over the temperature range from 25 °C to 600 °C, at a heating rate of 10 °C/min.

3. Results and discussion

3.1. Inhibition activity of CTS

Fig. 1 showed $O.D_{610nm}$ versus exposure time for CTS with *E. coli*. The time-inhibition studies demonstrated that CTS shows inhibition, which is related to its Mw. The $O.D_{610nm}$ values of tested samples are much less than blank control, especially for the CTS with Mw 50 kDa, and are stable with the increasing of time, which shows that CTS have a high rate of killing cells and high antibacterial activity. However, the addition of CTS of Mw 1000 kDa slightly increased the $O.D_{610nm}$ value at short culture times, which is perhaps attributed to its high viscosity.

In summary, CTS inhibit the growth of *E. coli*, but CTS of 50 kDa showed the most effective inhibition activity.

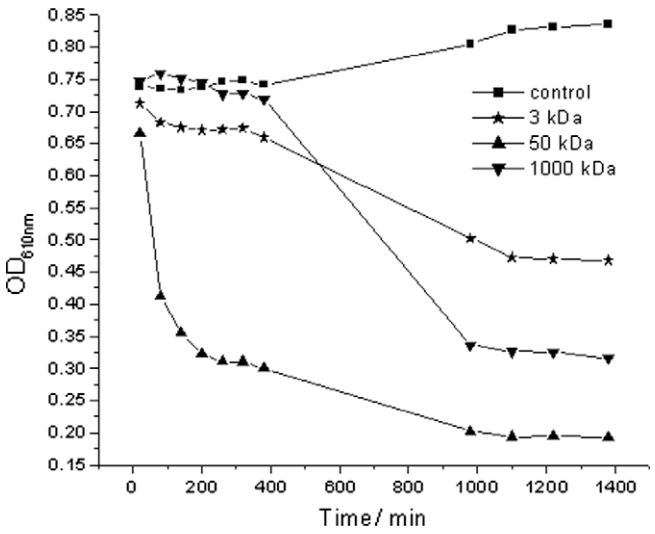

Fig. 1. The inhibitory effect of CTS on the growth of *E. coli*.

3.2. MIC

The MIC of CTS towards *E. coli* was examined at concentrations of 0.025–0.25% incubated for 24 h at 37 °C. The MIC values, ranging from 0.05% to 0.2%, slightly differed with the Mw of tested CTS. The MIC value of CTS with 50, 3 and 1000 kDa was 0.05%, 0.1% and 0.2%, respectively.

Seo et al. found the MIC of CTS for *E. coli* was 0.002% (Seo, Mitsuhashi, & Tanibe, 1992), Uchida et al. reported the MIC to be 0.025% (Uchida, Izume, & Ohtakara, 1989), Jeon et al. reported that MIC values of CTS were less than or equal to 0.06% against Gram-negative and gram-positive bacteria (Jeon et al., 2001). These reported MIC values are lower than our current results. This is probably due to differences in experimental methods, CTS characteristics, or medium pH applied as reported by Wang (1992).

3.3. Integrity of cell membrane

The cytoplasm and membrane of bacteria undoubtedly is the target for many inhibition agents. When bacterial membranes become compromised by interaction with inhibition agents, first, low molecular mass species such as K^+ and PO_4^{3-} tend to leach out, followed by DNA, RNA and other materials. These intracellular components are easily detected by the absorption at 260 nm as an indication of membrane damage (Chen & Cooper, 2002). So, by measuring the absorption at 260 nm and 280 nm, we can estimate the amount of DNA and proteins released from the cytoplasm, which is an indication of membrane damage.

The amount of DNA and RNA released from the *E. coli* suspension treated with CTS is shown in Fig. 2. The $O.D_{260nm}$ increased rapidly up to 40, 60, 100 min first followed by a gentle decrease, respectively for CTS of 3, 50 and 1000 kDa. All the three tested CTS have the abilities to damage the cell membrane of *E. coli* and make the nucleic acid released. It's obviously seen that the nucleic acid content induced by CTS of Mw 50 kDa was higher than others. The results indicated that CTS of Mw 50 kDa was most effective in damaging the cell membrane, which is in agreement with the results of the inhibition activity.

Jeon et al. found that both *E.coli* and *Staphylococcus aureus* treated with dimethylaminoethyl-chitosan (DMAEC), the release of 260 nm-absorbing materials quickly increased but the absorbance

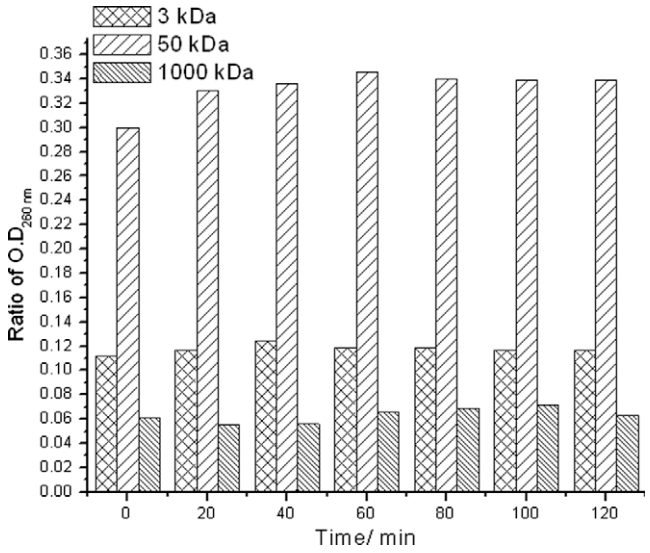

Fig. 2. The ratio release (vs. control) of cell materials absorbing at 260 nm from *E. coli* suspensions treated with CTS solutions.

value was different due to the difference in cell structures (Jeon & Kim, 2006), Liu et al. also found the same results (Liu et al., 2004).

The release of intracellular proteins from *E. coli* treated by CTS with different Mw is shown in Fig. 3. Initially, the absorption at 280 nm was markedly increased. When CTS of Mw 3 kDa mixed with *E. coli* suspension, the $O.D_{280nm}$ decreased up to 40 min later, which was perhaps due to the interaction of CTS with proteins from *E. coli* suspension. However, CTS of Mw 50 kDa mixed with *E. coli* suspension, the $O.D_{280nm}$ was reached maximum ($O.D_{280nm}$ 1.267) up to 40 min. When completely disrupted *E. coli* suspension of $O.D_{420}$ 0.8 by freeze–thaw repeat, the $O.D_{280nm}$ was 1.270, which indicated that CTS of Mw 50 kDa have already absolutely disrupted the cell membrane of *E. coli*, and the intracellular proteins were almost fully released. The results suggested that CTS could disrupt cell membrane and CTS with Mw 50 kDa appeared most effective leading to the release of intracellular materials, which is also consistent with the results of the inhibition activity.

3.4. OM permeability assays

For antibiotics to act on Gram-negative bacteria, first of all, them must enter into the plasmid space of through the outer membrane namely channels that shaped membrane protein (Zuo & Yao, 1998). *E. coli* possess representative Gram-negative bacterial membrane structure, which was composed of OM, IM and cytoplast space of inner- outer membrane. The OM situated outer of Gram-negative bacterial cell wall, and consist of some kinds of protein such as LPSs, phospholipid and lipoprotein and so on, and these are maintained together by electrostatic interactions with divalent cations that are required to stabilize the OM. Polycationic molecules could bind to the negatively charged O-specific oligosaccharide units of *E. coli* LPSs, thus disrupting the integrity of the OM and resulting in loss of the barrier function or blocking of the nutrient flow with concomitant bacterial death due to depletion of the nutrients. The permeability barrier of the OM is formed by cross-bridging involving lipid A molecules via divalent cation (magnesium and/or calcium). Compounds that bind either lipid A or divalent cation disrupt the organization of the OM and increase its permeability. Hence, the permeability of the bacterial OM was an important index to reflect whether drugs enter bacteria or not.

Alizarin red (AR) can interact with CTS and the CTS-AR complex is formed with the appearance of the new absorption peak at 530 nm. The decrease of absorbance at 422 nm and the increase of absorbance at 530 nm are in proportion to CTS concentration. Based on those above, a spectrophotometric method is proposed for the determination of CTS content. Between the absorption value of complex and the concentration of CTS have linear relationship when the concentration of Alizarin red and CTS was 3.1333×10^{-4} mol/L and 0–0.2 mg/ml, respectively (Gao et al., 2003). According to the equation:

$$y = 0.6378 - 1.1334x, R = 0.997$$

y and x are the absorption value at 422 nm and concentration of CTS (mg/ml), respectively.

Making the contents of 0 min as the initial concentration of CTS, the absorptivity of *E. coli* by CTS was considered as permeability degree.

The residual amounts of CTS after acted with *E. coli* were shown in Table 1. The amounts of CTS with Mw 3, 50 and 1000 kDa entered into cell suspension were different. The initial concentration of CTS was 0.1890 mg/ml, 0.1524 mg/ml and 0.1975 mg/ml, respectively. But, after an exposure of *E. coli* to CTS up to 30 min, the final concentration of CTS was 0.1798, 0.1260 and 0.1939 mg/ml, respectively. The changed values were 0.0092, 0.0264 and 0.0036 mg/ml each. So, the OM permeability was 4.90%, 17.3% and 1.82%. These data showed that CTS can alter the OM permeability, and different Mw CTS has different ability to alter the OM permeability. In the present study, just as above data shows, the affect CTS with Mw 50 kDa on membrane permeability was the strongest of all. The results also supported the fact that CTS of Mw 50 kDa is most effective against *E. coli*. So, CTS with Mw 50 kDa was selected for the following studies.

Helander found that CTS at pH 5.3 caused significant increases in a hydrophobic probe 1-N-phenylnaphthylamine (NPN) uptake compared with control cells, and that relatively high concentrations of CTS (250 ppm) were required to obtain significantly increased NPN uptake. These results suggested that CTS rapidly increase the permeability of the OM, which indicated OM is damaged and functionally invalid (Helander et al., 2001). Jeon also found that DMAEC rapidly increased NPN uptake via an increase in the permeability of OM (Jeon & Kim, 2006).

3.5. Transmission electron microscopy

In electron micrographs, the control showed an intact and apparent cell membrane as an electron dense line (Fig. 4a). However, CTS-treated *E. coli* showed badly disrupted and altered of cell membrane after 24 h, as depicted in Fig. 4b–d. CTS-treated *E. coli* cells had extra- and intracellular changes compared with the non-treated cells including a separation of the cytoplasmic membrane from the cell envelope and coagulation of the cytosolic components, and disruption of the OM structure with membrane sloughing and breaching, even disappeared. There were irregularly shaped and without membranes or cell wall on one side, and pores formed on the cell surface. Morphas of the bacteria were multiplicity, and the cells were markedly degraded from bacilliform to a spherical shape and irregularly condensed masses with bleb-like structures, with the loss of cell contents. The profile of bacterial became faint, the surface appeared burs, their structure turned dim and hollow, even perforated and crushed.

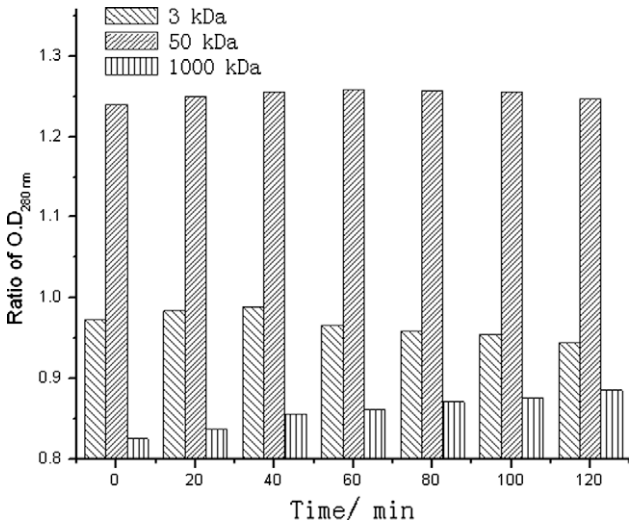

Fig. 3. The ratio release (vs. control) of cell materials absorbing at 280 nm from *E. coli* suspensions treated with CTS solutions.

Table 1
The residual concentration (mg/ml) of CTS after acted with *E. coli*.

Residual CTS (mg/mL)	Molecular weight of CTS		
Time (min)	3 kDa	50 kDa	1000 kDa
0	0.1890	0.1524	0.1975
30	0.1798	0.1260	1.1939

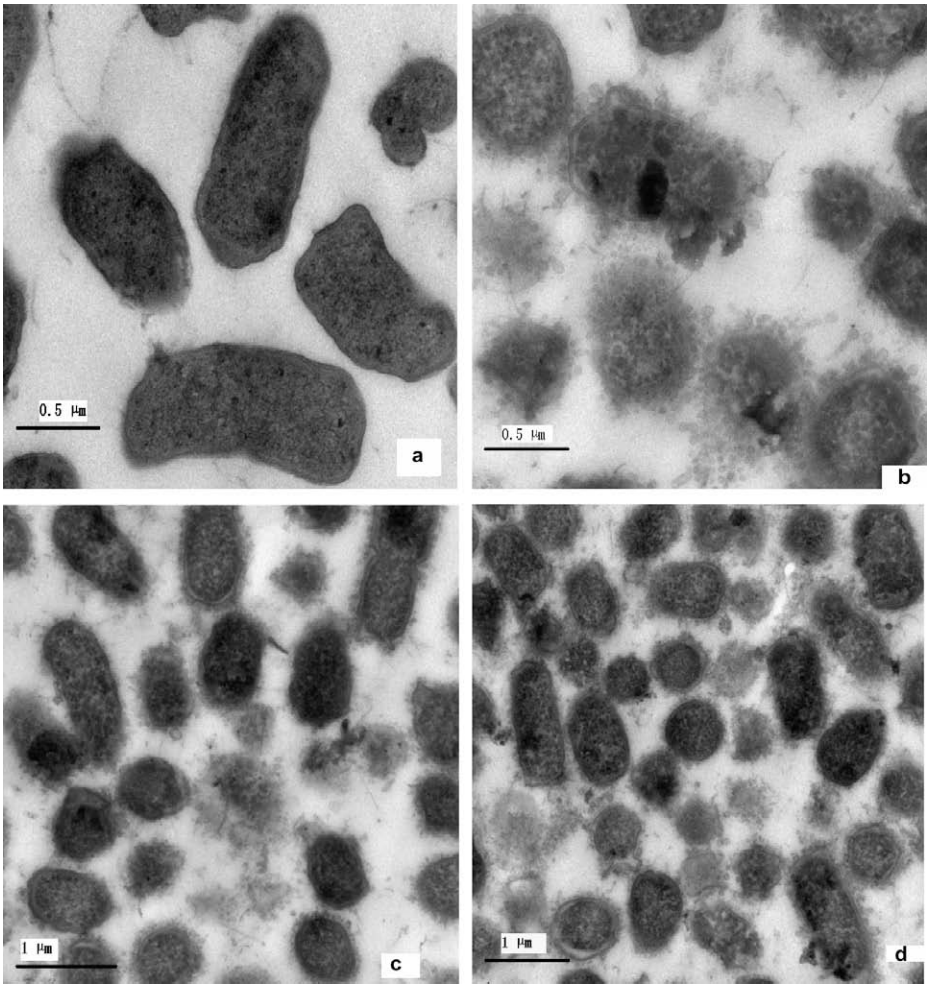

Fig. 4. TEM diagram of *E. coli* treated with (a) buffer and CTS with molecular 50 kDa (b–d), amplified 50,000, 50,000, 30,000 and 25,000, respectively. Bar:μm.

TEM studies showed that pore formation on the bacterial cell surface, indicated that it was lytic rather than static activity for CTS toward *E. coli*. These micrographs showed that CTS damaged the cell membranes of cells. Thus, membrane damage is the inhibition mechanism of CTS against *E coli*. Similar findings were reported by Helander et al. (2001) and Liu et al. (2004).

3.6. Determination of endoenzyme activity in the bacterial suspension

To prevent the intracellular components from releasing is one of the functions of cell wall. The LDH and γ-GT are endoenzymes, and could not leak to the outside of the bacteria. Hence, it could not measure their activities under normal situation. But, when the cell membrane or cell wall was damaged, endoenzyme would leak to the outside of the bacteria. Therefore, determinating the endoenzyme activity in the bacterial suspension can reflect the permeability change of cell membrane or cell wall (Hara & Yamakawa, 1995; Huang & Wang, 1996). The releasing of endoenzyme was proved by observing the enzyme activities of LDH and γ-GT in bacterial suspension, as was shown in Fig. 5. The addition of CTS resulted in a higher level of LDH and γ-GT activities than the control. The LDH as well as γ-GT activities were increased time dependently in *E. coli* suspensions. During the first 8 h, the LDH and γ-GT activities increased sharply except the control. Thereafter, they increased slightly, which means that CTS has permeability activity towards IM. Similar finding was reported by Liu et al. (2004) Jeon & Kim (2006).

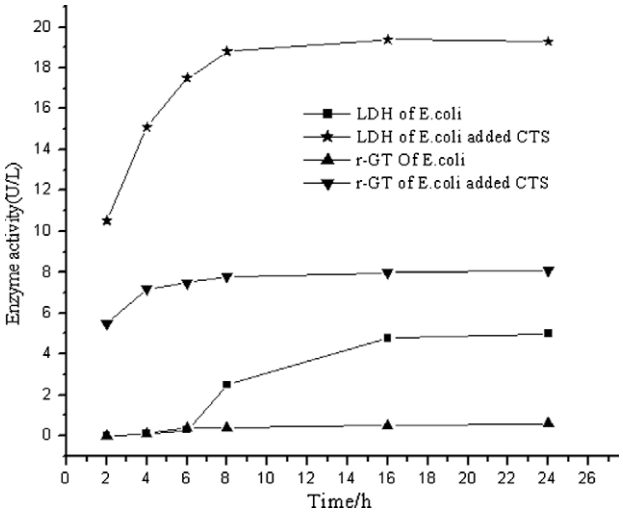

Fig. 5. Release of LDH and γ-GT in *E. coli* before and after treated with CTS.

Changes of cell membrane permeability due to the interaction of the microbial cell surface with CTS leading to the death of microbial cells have been proposed by various investigators (Chen & Chou, 2005; Leuba & Stossel, 1986; Tsai & Su, 1999; Young et al., 1982). In the present study, cell leakage of *E. coli* induced by CTS,

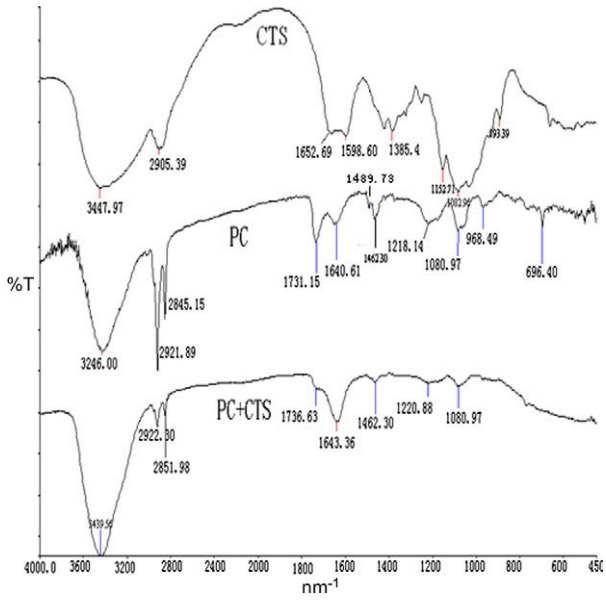

Fig. 6. FT-IR spectra of CTS, PC and CTS/PC complex.

OM permeability and determination of endoenzyme activity were studied. The results showed that CTS seriously damaged the cell membrane of *E. coli*, and accompanied with a lot of cellular contents released.

3.7. Interaction of CTS with phospholipid membrane

Structure of CTS, PC and PC/CTS complex were confirmed by FT-IR spectroscopy (Fig. 6). CTS show a distinct amide I band and amide II bands at 1598.60 and 1385.43 cm^{-1}, respectively. In this study, the intense absorption bands at the 1598.60 cm^{-1} indicated exist of $-NH_3^+$ (Williams & Fleming, 1980), but the band disappeared in PC/CTS complex. The IR spectrum of PC is also shown in Fig. 6, the most useful absorption bands are the —C=O stretching band, which appear in the 1750–1700 cm^{-1} region, and the phosphoryl group asymmetric and symmetric bands, which appear around 1240–1220 and 1085 cm^{-1}, respectively (Gomez-Fernandez & Villalain, 1998). In this study, the —C=O stretching band of PC liposomes appeared at 1731.15 cm^{-1}, which shifted to 1736.63 cm^{-1} in PC/CTS complex. Besides, the absorption band from stretching of —P=O appeared at 1218.14 cm^{-1}, which nearly disappeared in the PC/CTS complex.

The FT-IR spectrum of the PC/CTS complex exhibited most of the characteristic of CTS and PC but with some important differences. The disappearance of peaks at 1731.15 cm^{-1} from PC, 1598.60 and 1218.14 cm^{-1} from CTS, coupled with the new forming peak at the 1736.63 cm^{-1}, which suggests an attractive intermolecular interaction between CTS and PC have occured. Therefore, these results demonstrated that it was the carbonyl and phosphoryl group in PC that participated in the interaction with amino group in CTS. While Liu et al. thought that it was the phosphoryl group not carbonyl group in PC that participated in the interaction with CTS (Liu et al., 2004).

The thermal properties of CTS, PC and PC/CTS complex were analyzed by TG-DTA, as shown in Fig. 7. It was previously reported that TG curves showed weight loss of CTS took place in two stages (Qin, Du, & Xiao, 2002; Tirkistani, 1998). The first degradation stage of CTS was assigned to the loss of water (Bihari-Varga, Sepulchre, & Moczar, 1975), and the second stage as being due to the degradation of CTS, with the greatest weight loss occurring at 298 °C (Qin et al., 2002) or 334 °C with a weight loss of 49% (Tirkistani, 1998). However, in our study, TG and DTA curves indicated that CTS degraded in three stages. CTS starts to dehydrate at the very beginning of the heating , forming a weak peak at ~80 °C, which dehydrateated mostly a part of water itself (Bihari-Varga et al., 1975), CTS loses about 12.1% at heating to 80 °C, then the rate of the mass loss decreased. The second weight loss stage in TG of CTS starts at 240 °C and reaches a maximum at 380 °C with a weight loss of 48.4%, which corresponds to the depolymerization and decomposition of glucosamine units of CTS. The third step involved the decomposition (thermal and oxidative) of CTS and elimination of volatile products. The decrease in mass starts again and the additional value of mass loss at heating to 400 °C was of 37.5%. This result is similar to Maogen, Smith & Gorski (2004). From the second step to about 490 °C, there was a weak absorption peak appeared, which indicated that CTS yet not decomposed and oxidated fully. But for PC and PC/CTS, their weight loss showed two stages, the first were approximately at 50–200 °C and 50–150 °C with the loss rates of 7.8% and 8.9%, respectively. The second were at 250–400 °C and 230–380 °C with the loss rates of 70% and 58% each.

The DTA of the three samples revealed a major difference in their decomposition patterns. For DTA, the CTS and PC appeared three exothermic peaks, but the PC/CTS complex appeared two exothermic peaks. The peaks could not be accurately attributed, but the obvious differences between TG and DTA curves of PC/CTS complex, CTS and PC indicated that the interaction between CTS and PC have occurred, as was indicated by the FT-IR spectrum.

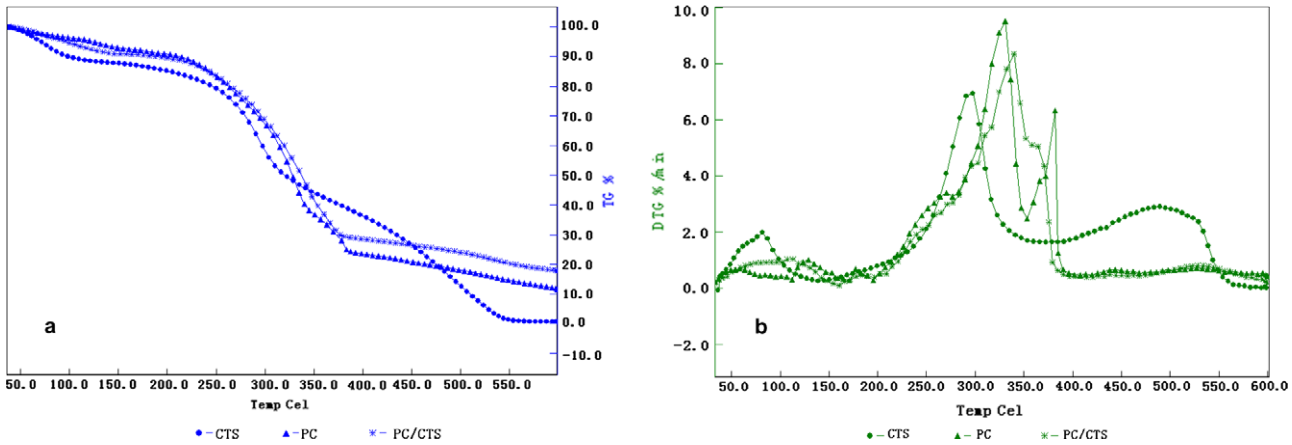

Fig. 7. Thermogravimetry (a) or differential thermogravimetry (b) curves of CTS, PC and PC/CTS.

4. Conclusions

In this study, the inhibition activity of three kinds of CTS with different Mw towards *E. coli* was studied in vitro. Experimental figures and data presented have provided several evidences that CTS demonstrated strong inhibition activity against *E. coli*, but the inhibition activity differed in Mw, within the tested CTS, the CTS with 50 kDa Mw possessed of the strongest inhibition activity. Ultrastructural analysis by TEM provided intuitionistic evidence that CTS caused cytoplasmic membrane disruption and lysis in *E. coli*. The integrity of cell membranes of *E. coli*, OM and IM permeability results suggested that CTS killed bacteria by disrupting the OM and IM of bacteria, which caused the damage of structure, function and permeability of *E. coli*, leakage of intracellular components and the ultimate lysis of cell. The FT-IR and TG-DTA analysis of PC/CTS complex was illuminated that CTS interacted with PC by forming new chemical bonds between the $-NH_2$ in CTS and the —C═O and —P═O groups in PC. These findings would provide further insight into the pharmaceutical application of inhibition medicament. An investigation with fluorescent labeled CTS with different Mw is under progress in our laboratory to find the exact action of CTS with bacteria.

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