Carbohydrate Polymers 86 (2011) 969–974

Contents lists available at ScienceDirect

Carbohydrate Polymers

journal homepage: www.elsevier.com/locate/carbpol

Effect of chitosan on membrane permeability and cell morphology of *Pseudomonas aeruginosa* and *Staphyloccocus aureus*

Yan Tao*, Li-Hong Qian, Jing Xie

College of Food Science and Technology, Shanghai Ocean University, Shanghai 201306, China

ARTICLE INFO

Article history:
Received 6 April 2011
Received in revised form 19 May 2011
Accepted 25 May 2011
Available online 6 June 2011

Keywords:
Chitosan
Antibacterial activity
Antibacterial mechanism
Membrane permeability

ABSTRACT

The mechanism of action of over 85% deacetylated chitosan on Gram-negative *Pseudomonas aeruginosa* and Gram-positive *Staphylococcus aureus* was investigated. Results showed that after treated with chitosan, the electric conductivity of bacteria suspensions increased, followed by increasing of the units of average release for alkaline phosphatase (ALP) and glucose-6-phosphate dehydrogenase (G6PDH). SDS-PAGE and agarose gel electrophoresis indicated that the soluble proteins and intact DNAs decreased or disappeared in chitosan-treated bacteria cells, demonstrating that chitosan performed its antibacterial function via increasing the permeability of cell membranes. Moreover, chitosan had a stronger effect on the cell membrane of *S. aureus* than on that of *P. aeruginosa* due to the differences in their cell structures. Transmission electron microscopy (TEM) observations further verified that *S. aureus* is more sensitive than *P. aeruginosa* to chitosan. Our works provide additional evidences in support of chitosan being regarded as a natural bactericide.

1. Introduction

Chitosan's antimicrobial activity has been well documented. Muzzarelli et al. (1990) examined N-carboxybutyl chitosan's antimicrobial activity against 298 pathogens, finding that it was particularly active against candidae and Gram-positive bacteria tested as determined by the agar dilution. When a thin pad of N-carboxybutyl chitosan was used, all candidae and most staphylococci were killed, whereas percentages of the bactericidal activities against most Gram-negative species were lower. However, no bactericidal activity was observed with Gram-positive streptococci and enterococci, and three Gram-negative *Salmonella* strains. Thus, it is likely that chitosan might be bactericidal or simply bacteriostatic, and each strain may be vulnerable/indifferent to the presence of chitosan. Recently, Wu et al. (2011) reported that they synthesized chitosan-poly(ε-caprolactone) (CPC) copolymers via an amino-group-protection method. The optimized quaternized CPCs (q-CPCs) were able to completely prevent growth of *Staphylococcus aureus* and *Escherichia coli*, as model Gram-positive and Gram-negative bacteria, respectively, at different concentrations of about 0.2% and 0.25%, respectively. At lower concentrations, these optimal q-CPCs had higher antibacterial activities against the both bacteria as compared to chitosan. Simultaneously, Vallapa et al. (2011) reported that the antibacterial activity of the surface-quaternized chitosan film against *S. aureus* and *E. coli* were superior to that of the virgin chitosan film. The other literature mentioned that chitosan displays antibacterial activities, with minimum inhibitory concentrations (MICs) reported to range from 100 to 10,000 mg L^{-1} against Gram-negative bacteria (Helander, Nurmiaho-Lassila, Ahvenainen, Rhoades, & Roller, 2001), and from 100 to 1250 mg L^{-1} against Gram-positive bacteria (Bae, Jun, Lee, Paik, & Kim, 2006; Jeon, Park, & Kim, 2001; No, Park, Lee, & Meyers, 2002; Vishu Kumar, Varadaraj, Lalitha, & Tharanathan, 2004).

It has been demonstrated that chitosan's antibacterial activity depends on the strain examined and on its growth phase, besides chitosan concentration, molecular weight, degree of deacetylation, and pH, temperature, and composition of the medium (Chen & Chou, 2005; Chung, Wang, Chen, & Li, 2003; Liu et al., 2006; Muzzarelli et al., 1990; No et al., 2002). Because it has been tested under widely varied conditions, it is hard to compare chitosan's antibacterial effect among results obtained by different researchers.

Several antimicrobial mechanisms have been proposed for chitosan and its soluble derivatives. In the first, the cationic chitosans interact with anionic moieties at the cell surface thereby altering cell permeability and resulting in material being prevented from entering the cell and/or material being leaked from the cell (Chen, Liau, & Tsai, 1998; Jung, Kim, Choi, Lee, & Kim, 1999). The second mechanism involves the binding of chitosan with DNA to inhibit RNA synthesis, which occurs through chitosan penetration toward the nuclei of the microorganisms and interference with the synthesis of mRNA and proteins (Rabea, Badawy, Stevens, Smagghe,

* Corresponding author. Tel.: +86 21 61900384; fax: +86 21 61900365.
E-mail address: ytao@shou.edu.cn (Y. Tao).

0144-8617/$ – see front matter.
doi:10.1016/j.carbpol.2011.05.054

& Steurbaut, 2003; Wang, Du, & Liu, 2004). In another proposed mechanism, chitosan also acts as a chelating agent that selectively binds trace transition metals and thereby inhibits the production of toxins and microbial growth (Cuero, Osuji, & Washington, 1991). Various techniques have been applied to *S. aureus* and *E. coli*, to investigate and compare chitosan's antibacterial modes (Vishu Kumar, Varadaraj, Gowda, & Tharanathan, 2005; Xing et al., 2009). However, up to the present, there are few reports on chitosan's antibacterial activity and antibacterial mechanism against bacteria in the family Pseudomonadaceae. These bacteria are considered psychrotrophs, growing well at 0–15 °C, thereby being regarded as the most important spoilage bacteria originating in refrigerated food (Jay, 2000; Vela, 1997). Pseudomonads, especially *Pseudomonas aeruginosa*, can also cause outbreaks of intramammary infections. So far, although the activity and interactions of some antibiotics, innate defense molecules such as antimicrobial peptides and chemicals against *P. aeruginosa* were reported (Bouhdid, Abrini, Zhiri, Espuny, & Manresa, 2009; Bellemare, Vernoux, Morin, Gaqné, & Bourbonnais, 2010; Jayaraman, Sakharkar, Lim, Tanq, & Sakharkar, 2010; Uccelletti et al., 2010), there is no direct evidence for the mode of action of chitosan on *P. aeruginosa*.

The objectives of the present study were to elucidate the interaction between chitosan and Gram-negative *P. aeruginosa*. In addition, Gram-positive *S. aureus* was also selected as a tested strain to compare the modes of action of chitosan on the both strains.

2. Materials and methods

2.1. Materials and microorganisms

Chitosan (source: crab shell, high MW, degree of deacetylation > 85%) was purchased from Li Zhong Chitin Company (Qingdao, China). Strains of *P. aeruginosa* (ATCC27853), *S. aureus* (ATCC27853), *E. coli* (ATCC25922), *Listeria monocytogenes* (ATCC19115), *Salmonella enteritidis* (ATCC13076) and *Bacillus cereus* (ATCC14579) were obtained from the American Type Culture Collection (ATCC). Sodium dodecyl sulfate (SDS), acrylamide, N, N′-methylenebisacrylamide, *o*-nitrophenyl-β-D-galactoside (ONPG) and glutaraldehyde were purchased from Sigma Chemicals Company (St. Louis, Mo). All other reagents were of the highest grade available commercially.

2.2. Determination of minimum inhibitory concentrations (MICs) and minimum bactericidal concentrations (MBCs)

MICs were determined by microtiter broth dilution method, following the guidelines in the literature (Andrews, 2001). In brief, inocula of those strains were prepared by adjusting overnight cultures to containing 2×10^5 CFU mL^{-1} in nutrient broth. Aliquots of 50 μL were mixed with chitosan (dissolved in 0.5% acetic acid, and adjusted to pH 6.0 with 0.1 M NaOH) of 50 μL of serial two-fold dilutions in a 96-well plate, and the plate was incubated with shaking at 37 °C for 18 h. MIC was defined as the lowest concentration of chitosan where no growth was observed by microscopic examination. On the other hand, 10 μL mixtures from the wells with no growth were spread on agar plates for MBC determination. MBC was defined as the lowest concentration of chitosan where no colony was observed on agar plates after 48 h incubation at 37 °C.

2.3. Electric conductivity assay

After incubated at 37 °C for overnight, *S. aureus* and *P. aeruginosa* were harvested by centrifugation at 11,000 × *g* for 10 min. The pellets were washed and resuspended in 0.1 M phosphate buffer (pH 7.4). The final cell suspensions were adjusted to 10^8 CFU mL^{-1} and mixed with chitosan (600 mg L^{-1} in 0.5% acetic acid, pH 6.0) to final chitosan concentrations of 150 and 300 mg L^{-1}, and then the mixtures were incubated at room temperature and measured for their electric conductivity every 10 min for 2 h. The experiment without chitosan was used as blank control.

2.4. Alkaline phosphatase (ALP) and glucose-6-phosphate dehydrogenase (G6PDH) assay

The chitosan (600 mg L^{-1} in 0.5% acetic acid, pH 6.0) was added to a 0.85% (w/w) NaCl solution containing *S. aureus* or *P. aeruginosa* at 10^7 CFU mL^{-1} to a final concentration of 300 mg L^{-1}. After incubated at 37 °C for 8 h, a 0.2-mL aliquot of the cell suspension was withdrawn and added to the reaction mixture.

ALP activity was determined using the method described by Malamy and Horecker (1964). The reaction mixture (total volume: 1 mL) contained 0.1 mg *p*-nitrophenylphosphate in 0.5 M Tris–HCl buffer (pH 8). The reaction was followed at 28 °C by measuring the optical density of the suspension at 420 nm. A unit of released ALP activity was defined as the amount of enzyme that produced 1 μM of *p*-nitrophenol-equivalent in 1 min at 28 °C.

G6PDH activity was determined using the method described by Malamy and Horecker (1964). A 0.2-mL aliquot of the cell suspension as described in the above paragraph was added to the reaction mixture. G6PDH activity was determined in a solution (total volume: 1 mL) containing 0.05 M Tris-HCl (pH 8), 0.01 M $CaCl_2$, 1.0 μM glucose-6-phosphate and 0.4 μM triphosphopyridine nucleotide (TPN). The reaction was followed at 28 °C by measuring the optical density of the suspension at 340 nm. A unit of released G6PDH activity was defined as the amount of enzyme that reduced 1 μM of TPN-equivalent in 1 min at 28 °C.

2.5. Inner membrane (IM) permeabilization assay

Inner membrane permeabilization was determined by measuring the release of cytoplasmic β-galactosidase activity from *E. coli* into the culture medium using ONPG as the substrate (Ibrahim, Sugimoto, & Aoki, 2000). Logarithmic-phase bacteria grown in nutrient broth containing 2% lactose was harvested by centrifugation at 11,000 × *g* for 10 min, washed and resuspended in 0.5% NaCl solution. The final cell suspension was adjusted to an absorbance of 1.2 at 420 nm. A 1.6-mL aliquot was mixed with chitosan (600 mg L^{-1} or 300 mg L^{-1} in 0.5% acetic acid, pH 6.0) of 1.6 mL and 30 mM ONPG of 150 μL. The production of *o*-nitrophenol over time was determined by monitoring the increase in absorbance at 420 nm using a spectrophotometer. The control contained a 1.6-mL aliquot described as above and 0.5% acetic acid (pH 6.0) of 1.6 mL and 30 mM ONPG of 150 μL.

2.6. SDS-PAGE and DNA fragmentation

Logarithmic phase cells of *S. aureus* and *P. aeruginosa* (~10^7 CFU mL^{-1}) in nutrient broth were mixed, respectively with chitosan (600 mg L^{-1} in 0.5% acetic acid, pH 6.0) to a final chitosan concentration of 300 mg L^{-1}, and then incubated at room temperature. Aliquots of 5 mL were withdrawn every 1 h for 3 h and 6 h for *S. aureus* and *P. aeruginosa*, respectively, and then centrifuged at 11,000 × *g* for 10 min. The pellet was subjected to SDS-PAGE analysis according to Laemmli (1970). The SDS-PAGE was performed with a 4% stacking gel and a 10% separating gel. Proteins bands were stained with 0.1% Coomassie Brilliant Blue R-250 and destained in 25% (v/v) of ethanol and 9% (v/v) of acetic acid.

For DNA fragmentation analysis, aliquots of 5 mL as described in the above paragraph were withdrawn after incubated for 1 h and 8 h, and subjected to centrifugation at 11,000 × *g* for 10 min. DNAs

Table 1
Minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) of chitosan against the six types of strain.

Bacteria	MIC (mg mL^{-1})	MBC (mg mL^{-1})
S. aureus	0.38	0.75
E. coli	0.75	0.75
L. monocytogenes	3	>3
S. enteritidis	3	300
B. cereus	3	>3
P. aeruginasa	3	>3

were extracted from the pellets using Kit (Tiangen Co., Beijing, China) and were run on a 1% agarose gel.

2.7. Transmission electron microscopy (TEM)

TEM analysis was performed following the guidelines in the literature with slight modification (Li, Wu, Wang, & Sun, 2002). Overnight cultures of *S. aureus* and *P. aeruginosa* were centrifuged and the pellets were washed and suspended in 0.1 M sodium phosphate buffer (pH 7.4) to an absorbance of 0.4 at 600 nm. The cell suspensions were incubated with chitosan (final concentration 300 mg L^{-1}) at 37 °C for 20 min and 2 h, and then centrifuged at 11,000 × *g* for 10 min. The resulting pellets were subjected to a series of treatments according to the guidelines in the literature to perform TEM analysis.

2.8. Statistic analysis

All experiments were carried out in triplicate, and average values with standard deviation were revealed. Significant differences between the 2 groups were examined using *t*-test. A P value < 0.05 denoted the presence of a statistically significant difference.

3. Results and discussion

3.1. Minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) of chitosan against the six types of strain

The antimicrobial activity of chitosan was quantitatively assessed against three Gram-positive and three Gram-negative bacteria by determining the MIC and MBC. As shown in Table 1, in the case of Gram-positive bacteria, MIC value of chitosan was 3 mg mL^{-1} against both *L. monocytogenes* and *B. cereus* and 0.38 mg mL^{-1} against *S. aureus*. In the case of Gram-negative bacteria, MIC value was 3 mg mL^{-1} against both *S. enteritidis* and *P. aeruginosa* and 0.75 mg mL^{-1} against *E. coli*. MBCs of chitosan against six types of strain were 0.75 mg mL^{-1}, 3 mg mL^{-1}, or higher. Chitosan was thought to have antibacterial activities only at acidic pH because of its poor solubility at pH > 6.5 (Rabea et al., 2003). Our results using over 85% deacetylated chitosan, soluble in 0.5% acetic acid (pH 6.0), indicated that chitosan at acidic pH significantly inhibited the growth of three Gram-positive and three Gram-negative bacteria in 18 h at concentrations ranged from 0.38 to 3 mg mL^{-1}. Inhibition with 5000 ppm acetic acid (pH was adjusted to 6.0) was negligible. Thus, using acetic acid at low concentration for chitosan did not have any additional adverse effect on bacterial growth. Many literatures reported the IMC values of chitosan with different ranges. Because the bacterial strains and the experimental conditions in this study are different from those in the literature, it is difficult to directly compare chitosan's antibacterial effect with results obtained by other researchers.

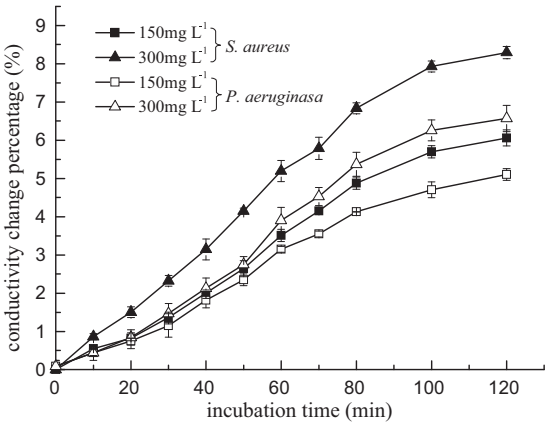

Fig. 1. Electric conductivity of cell suspensions for chitosan-treated *S. aureus* and *P. aeruginosa*.

3.2. Electric conductivity of cell suspensions for chitosan-treated S. aureus and P. aeruginosa

We determined electric conductivity of the cell suspensions for *S. aureus* and *P. aeruginosa* treated with 150 and 300 mg L^{-1} chitosan. As shown in Fig. 1, the electric conductivity showed a time-dependent increasing manner. It was found that the electric conductivity of suspensions for *S. aureus* and *P. aeruginosa* began to increase after treated with 150 and 300 mg L^{-1} chitosan for 10 min, and when the incubation time was 2 h, the electric conductivity increased by 6.1% and 8.3% for *S. aureus*, and 5.1% and 6.6% for *P. aeruginosa* compared with the controls, showing that 300 mg L^{-1} chitosan was more effective than 150 mg L^{-1} chitosan. On the other hand, differences in the changes of the electric conductivity between *S. aureus* and *P. aeruginosa* also suggested that the effect of chitosan on the membrane permeability of *S. aureus* was greater than on that of *P. aeruginosa*.

3.3. Effect of chitosan on the leakage of enzymes from S. aureus and P. aeruginosa

The effects of chitosan on the leakage of ALP and G6PDH from *S. aureus* and *P. aeruginosa* were shown in Table 2. The units of average release for both ALP and G6PDH in the cell suspension of *S. aureus* were higher compared with those in the cell suspension of *P. aeruginosa*. The control, which consisted of bacteria in a 0.5% acetic acid solution, showed zero units of the both enzymes released (data not shown). We also observed that the release of enzymes reached a plateau in 2 h for ALP and in 5 h for G6PDH. Malamy and Horecker (1964) reported that ALP was an extracellular enzyme, while G6PDH was found in the cell membrane. The locations of these two enzymes clearly provided the answer as to why ALP was the first molecule to be released into the medium, followed by G6PDH.

3.4. SDS-PAGE patterns of cellular soluble proteins from chitosan-treated S. aureus and P. aeruginosa

As shown in Fig. 2, the normal cell protein electrophoresis bands of *S. aureus* and *P. aeruginosa* appeared strong and clear. After

Table 2
Effect of chitosan on the leakage of enzymes from *S. aureus* and *P. aeruginosa*.

	Alkaline phosphatase (Units)	G-6-P dehydrogenase (Units)
S. aureus	128.4 ± 13.6	185.7 ± 17.4
P. aeruginasa	98.2 ± 11.7	147.8 ± 14.3

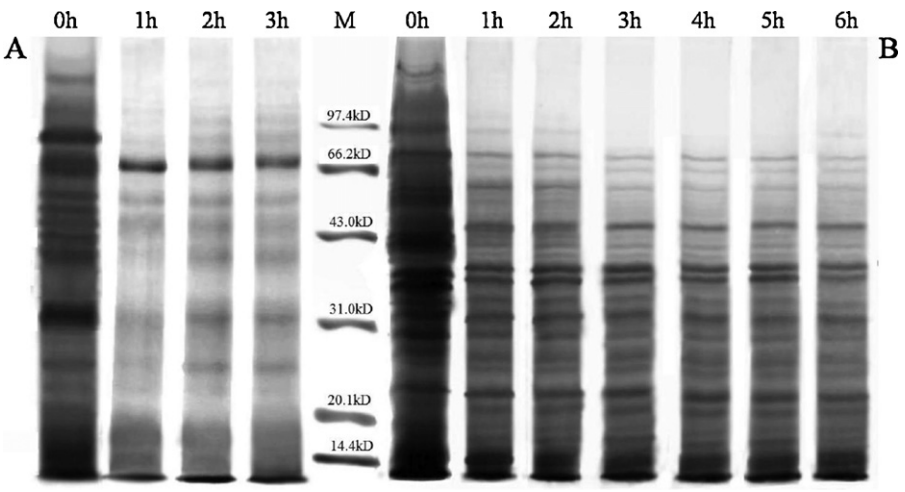

Fig. 2. SDS-PAGE patterns of cellular soluble proteins from chitosan-treated *S. aureus* (A) and *P. aeruginosa* (B).

treated with 300 mg L^{-1} chitosan for 1–3 h, bands of all-molecular-weight proteins for *S. aureus* appeared obviously shallow, even disappearing altogether. In the case of *P. aeruginosa*, the protein contents of chitosan-treated cells reduced, and as the incubation time increased (after 3 h), these characteristics became more distinct. On the other hand, protein contents in the cell-free supernatant increased compared with control culture supernatants (data not shown). These results suggested that chitosan decreased the content of cellular soluble proteins by permeating and disrupting cell membranes. However, the effect of chitosan on membrane permeability of *S. aureus* was more obvious than on that of *P. aeruginosa*. Cui et al. (2009) proposed that chitosan probably inhibited protein synthesis or control gene expression. The mechanism of protein breakdown remains unclear.

3.5. Agarose gel electrophoresis of DNAs from chitosan-treated S. aureus and P. aeruginosa

The release of the intracellular component, DNA, is also an indication of membrane damage. DNAs isolated from *S. aureus* and *P. aeruginosa* treated with 300 mg L^{-1} chitosan for 1 h and 8 h were analyzed by agarose gel electrophoresis. As shown in Fig. 3, specific DNA degradative smearing typical of necrotic degeneration was prominent in *P. aeruginosa* cells, especially for cells treated with chitosan for 8 h. In contrast, the occurrence of DNA disappearing was observed with chitosan-treated *S. aureus* cells even at time of 1 h. These shifts occurring on *S. aureus* and *P. aeruginosa* prompted that chitosan seems to be more active against *S. aureus* than against *P. aeruginosa*.

3.6. Release of cytoplasmic β-galactosidase from E. coli treated with chitosan

The inner layer of Gram-negative bacteria consists of phosphatidyl glycerol and cardiolipin (Je & Kim, 2006). The cytoplasmic β-galactosidase is released as a consequence of change in inner membrane permeability. In this study, we used model *E. coli* since it is known that the ability of chitosan to permeate the *E. coli* inner membrane can be verified as a function of cytoplasmic β-galactosidase release, with bacteria grown in lactose-containing medium. As shown in Fig. 4, when cells were treated with two different concentrations of chitosan, a lag time of about 10 min was followed by a progressive release of the cytoplasmic β-galactosidase for up to 90 min to reach a steady state. However, after treated for 50 min, the effect of 300 mg L^{-1} chitosan on

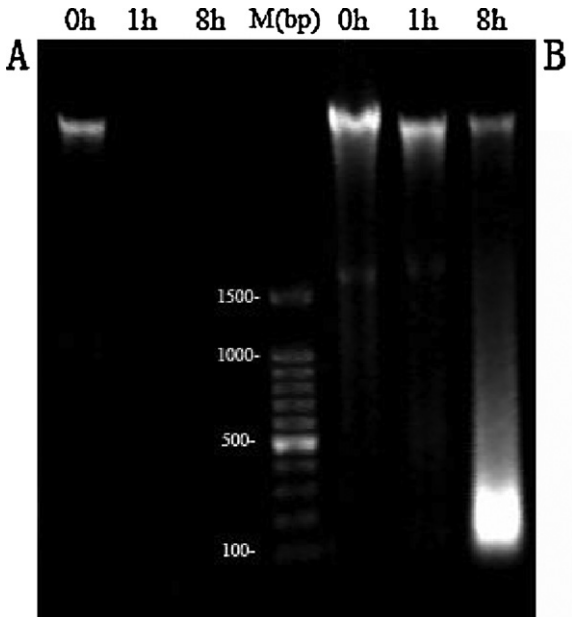

Fig. 3. Agarose gel electrophoresis of DNAs extracted from chitosan-treated *S. aureus* (A) and *P. aeruginosa* (B).

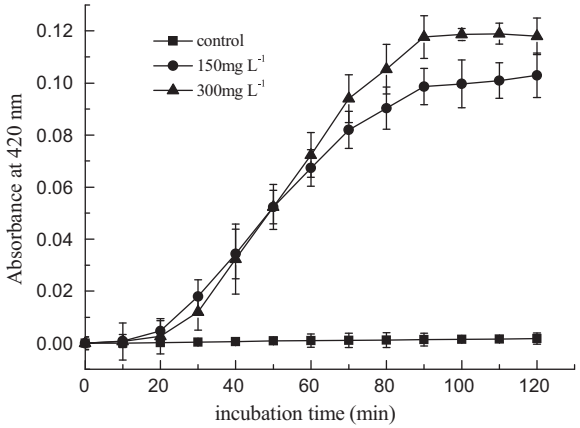

Fig. 4. Release of cytoplasmic β-galactosidase from *E. coli* treated with chitosan.

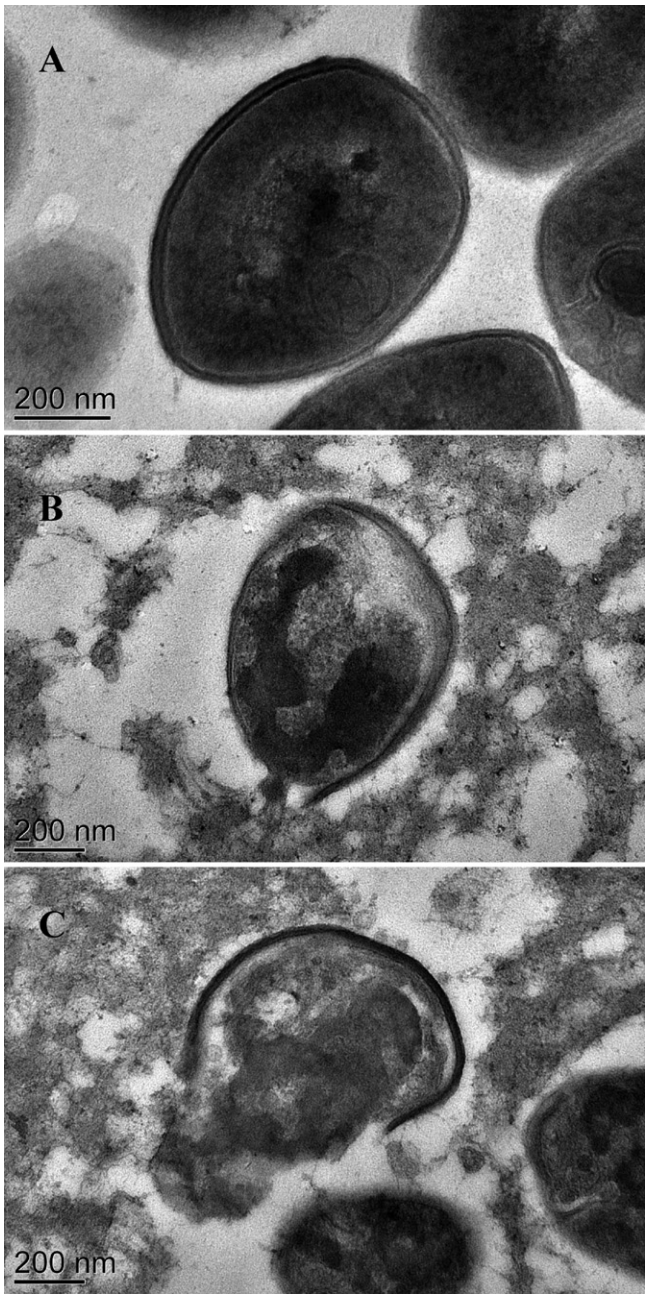

Fig. 5. Transmission electron micrographs of *S. aureus* treated with chitosan for 0 min (A), 20 min (B) and 2 h (C).

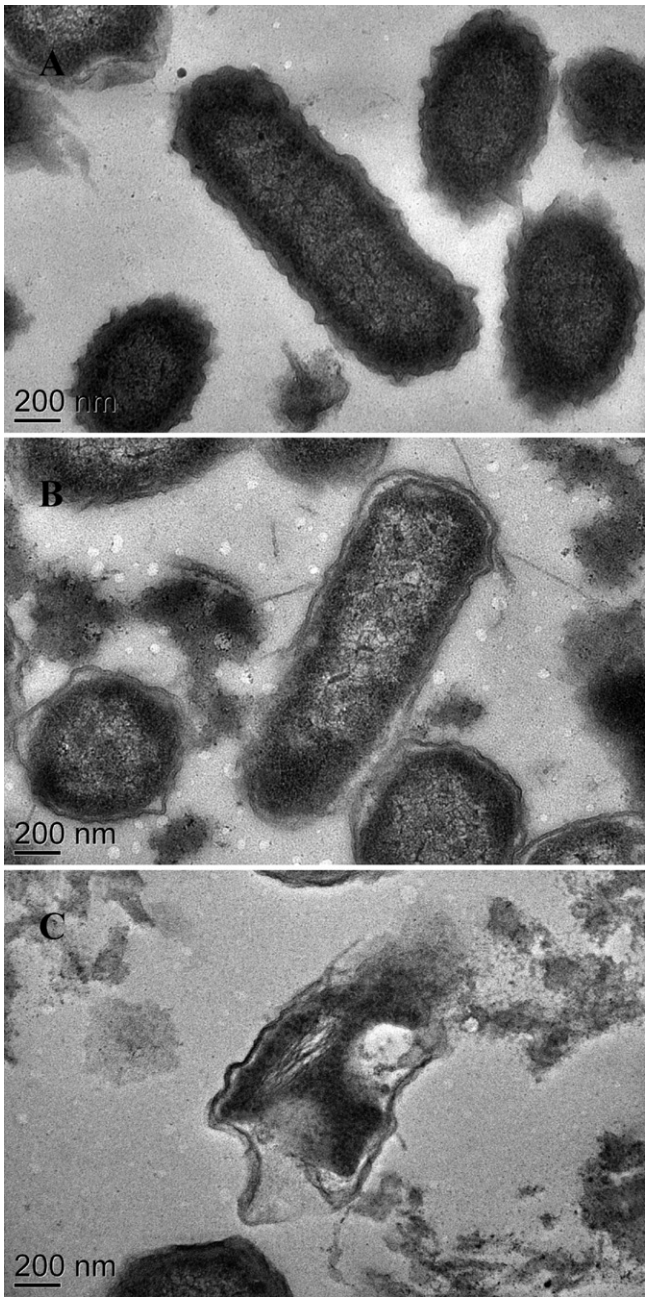

Fig. 6. Transmission electron micrographs of *P. aeruginosa* treated with chitosan for 0 min (A), 20 min (B) and 2 h (C).

the membrane permeability was greater than that of 150 mg L^{-1} chitosan. These results indicated that the release of cytoplasmic β-galactosidase caused by chitosan was time- and dose-dependant, which was coincident with the results reported by Liu, Du, Wang, and Sun (2004) and Xing et al. (2009). Thus, it is reasonable to consider that chitosan can also increase the inner membrane permeability of the Gram-negative *P. aeruginosa*.

3.7. Transmission electron micrographs (TEM) of S. aureus and P. aeruginosa treated with chitosan

The effects of chitosan on the cell morphology of *S. aureus* and *P. aeruginosa* were investigated by TEM. The electron micrographs showed that normal cells were surrounded by the cell membranes with compact surface, without release of intracellular components and notable ruptures on the cell surfaces (Figs. 5A and 6A). As compared to the untreated controls, remarkable modifications of bacterial cell membranes were found after a period of exposure to 300 mg L^{-1} chitosan. As shown in Figs. 5B and 6B, when bacterial suspensions were exposed to chitosan for 20 min, some ruptures, which could cause some slight leakages of cellular cytoplasmic contents, were observed on the cell membranes of *S. aureus* and *P. aeruginosa*, but the morphous of cells still were regular. Two hours of treatment later, the cytoplasmic membranes of chitosan-treated cells completely collapsed (Figs. 5C and 6C). However, it was noted that the adhering of chitosan to *S. aureus* was greater than to *P. aeruginosa* in amount.

The above results effectively demonstrated that chitosan could interact more strongly with Gram-positive *S. aureus* than with Gram-negative *P. aeruginosa*. Similar observations have also been reported by other researchers for chitosan-treated *E. coli* and *S.*

aureus. Such results are probably attributed to the differences in their membrane structures. All Gram-negative bacteria possess an outer membrane (OM) due to the presence of lipopolysaccharide (LPS) molecule, and outer membrane plays a role as a drug barrier (Nikaido, 1996). But the peptidoglycan layer of the cell wall of Gram-positive *S. aureus* is composed of networks with plenty of pores, so foreign molecules can enter the cell without difficulty (Xing et al., 2009). Jeon et al. (2001) reported that 89% deacetylated chitosan had more effective activity against pathogens than against non-pathogens expect in the case of lactic acid bacteria. Here our data supported this conjecture.

4. Conclusions

The results reported here demonstrated that chitosan's antibacterial activities against *S. aureus* and *P. aeruginosa* are due to the interactions of this polycation with cell membranes, consequently increasing the membrane permeability. These alterations occurring on the membrane structures resulted in the leakage of cell inclusions (enzymes, nucleotides, proteins and ions) from *S. aureus* and *P. aeruginosa*. The bulk of evidence indicated that chitosan had a stronger effect on the cell membrane of *S. aureus* than on that of *P. aeruginosa* due to their differences in the structures of cell membranes. The antibacterial mechanism of chitosan against *P. aeruginosa* seemed to be similar to that against *E. coli*. Our works provide additional evidences in support of chitosan being regarded as a natural bactericide.

Acknowledgements

This work was supported by a Grant-in-Aid for Program of Shanghai Subject Chief Scientist from the Science and Technology Commission of Shanghai Municipality (No. 09XD1402000) and Leading Academic Discipline Project from Shanghai Municipal Education Commission (No. J50704).

References

Andrews, J. M. (2001). Determination of minimum inhibitory concentrations. *Journal of Antimicrobial Chemotherapy*, *48*, 5–16.

Bae, K., Jun, E. J., Lee, S. M., Paik, D. I., & Kim, J. B. (2006). Effect of water-soluble reduced chitosan on *Streptococcus mutans*, plaquere growth and biofilm vitality. *Clinical Oral Investigations*, *10*, 102–107.

Bellemare, A., Vernoux, N., Morin, S., Gaqné, S. M., & Bourbonnais, Y. (2010). Structural and antimicrobial properties of human pre-elafin/trappin-2 and derived peptides against *Pseudomonas aeruginosa*. *BMC Microbiology*, *10*, 253.

Bouhdid, S., Abrini, J., Zhiri, A., Espuny, M. J., & Manresa, A. (2009). Investigation of functional and morphological changes in *Pseudomonas aeruginosa* and *Staphylococcus aureus* cells induced by *Origanum compactum* essential oil. *Journal of Applied Microbiology*, *106*, 1558–1568.

Chen, C. S., Liau, W. Y., & Tsai, G. J. (1998). Antibacterial effects of N-sulfonated and N-sulfobenzoyl chitosan and application to oyster preservation. *Journal of Food Protection*, *61*, 1124–1128.

Chen, Y. L., & Chou, C. C. (2005). Factors affecting the susceptibility of *Staphylococcus aureus* CCRC 12657 to water soluble lactose chitosan derivative. *Food Microbiology*, *22*, 29–35.

Chung, Y. C., Wang, H. L., Chen, Y. M., & Li, S. L. (2003). Effect of abiotic factors on the antibacterial activity of chitosan against waterborne pathogens. *Bioresource Technology*, *88*, 179–184.

Cuero, R. G., Osuji, G., & Washington, A. (1991). N-carboxymethyl chitosan inhibition of aflatoxin production: Role of zinc. *Biotechnology Letters*, *13*, 441–444.

Cui, W. H., Quan, X., Tao, K., Teng, Y., Zhang, X. G., Liu, Y., et al. (2009). Mechanism of action of neomycin on *Erwinia carotovora* subsp. *carotovora*. *Pesticide Biochemistry and Physiology*, *95*, 85–89.

Helander, I. M., Nurmiaho-Lassila, E. L., Ahvenainen, R., Rhoades, J., & Roller, S. (2001). Chitosan disrupts the barrier properties of the outer membrane of Gram-negative bacteria. *International Journal of Food Microbiology*, *71*, 235–244.

Ibrahim, H. R., Sugimoto, Y., & Aoki, T. (2000). Ovotransferrin antimicrobial peptide (OTAT-92) kills bacteria through a membrane damage mechanism. *Biochimica et Biophysica Acta*, *1523*, 196–205.

Jay, J. M. (2000). *Modern food microbiology* (6th ed.). Gaithersburg: Aspen Publishers, Inc.

Jayaraman, P., Sakharkar, M. K., Lim, C. S., Tanq, T. H., & Sakharkar, K. R. (2010). Activity and interactions of antibiotic and phytochemical combinations against *Pseudomonas aeruginosa* in vitro. *International Journal of Biological Sciences*, *6*, 556–568.

Je, J. Y., & Kim, S. K. (2006). Chitosan derivatives killed bacteria by disrupting the outer and inner membrane. *Journal of Agricultural and Food Chemistry*, *54*, 6629–6633.

Jeon, Y. J., Park, P. J., & Kim, S. K. (2001). Antimicrobial effect of chitooligosaccharides produced by bioreactor. *Carbohydrate Polymers*, *44*, 71–76.

Jung, B. O., Kim, C. H., Choi, K. S., Lee, Y. M., & Kim, J. J. (1999). Preparation of amphiphilic chitosan and their antimicrobial activities. *Journal of Applied Polymer Science*, *72*, 1713–1719.

Laemmli, U. K. (1970). Cleavage of structural proteins during the assembly of the bacteriophage T4. *Nature*, *227*, 680–685.

Li, J. E., Wu, W. L., Wang, Z. Y., & Sun, G. L. (2002). Apoptotic effect of As_2S_2 on K562 cells and its mechanism. *Acta Pharmacologica Sinica*, *23*, 991–996.

Liu, H., Du, Y. M., Wang, X. H., & Sun, L. P. (2004). Chitosan kills bacteria through cell membrane damage. *International Journal of Food Microbiology*, *95*, 147–155.

Liu, N., Chen, X. G., Park, H. J., Liu, C., Liu,. G., Meng, C. S., et al. (2006). Effect of MW and concentration of chitosan on antibacterial activity of *Escherichia coli*. *Carbohydrate Polymers*, *64*, 60–65.

Malamy, M. H., & Horecker, B. L. (1964). Release of alkaline phosphatase from cells of *Escherichia coli* upon lysozyme spheroplast formation. *Biochemistry*, *3*, 1889–1893.

Muzzarelli, R., Tarsi, R., Filippini, O., Giovanetti, E., Biagini, G., & Varaldo, P. E. (1990). Antimicrobial properties of N-carboxybutyl chitosan. *Antimicrobial Agents and Chemotherapy*, *34*, 2019–2023.

Nikaido, H. (1996). *Cellular and molecular biology*. Washington: ASM Press.

No, H. K., Park, N. Y., Lee, S. H., & Meyers, S. P. (2002). Antibacterial activity of chitosans and chitosan oligomers with different molecular weights. *International Journal of Food Microbiology*, *74*, 65–72.

Rabea, E. I., Badawy, M. E.-T., Stevens, C. V., Smagghe, G., & Steurbaut, W. (2003). Chitosan as antimicrobial agent: Applications and mode of action. *Biomacromolecules*, *4*, 1457–1465.

Uccelletti, D., Zanni, E., Marcellini, L., Palleschi, C., Barra, D., & Manqoni, M. L. (2010). Anti-*Pseudomonas* activity of frog skin antimicrobial peptides in a *Caenorhabditis elegans* infection model: A plausible mode of action in vitro and in vivo. *Antimicrobiological Agents Chemotherapy*, *54*, 3853–3860.

Vallapa, N., Wiarachai, O., Thongchul, N., Pan, J. S., Tangpasuthadol, V., Kiatkamjornwong, S., et al. (2011). Enhancing antibacterial activity of chitosan surface by heterogeneous quaternization. *Carbohydrate Polymers*, *83*, 868–875.

Vela, G. R. (1997). *Applied food microbiology*. Belmont: Star Press.

Vishu Kumar, A. B., Varadaraj, M. C., Lalitha, R. G., & Tharanathan, R. N. (2004). Low molecular weight chitosans: Preparation with the aid of papain and characterization. *Biochimica et Biophysica Acta*, *1670*, 137–146.

Vishu Kumar, A. B., Varadaraj, M. C., Gowda, L. R., & Tharanathan, R. N. (2005). Characterization of chito-oligosaccharides prepared by chitosan olysis with the aid of papain and pronase and their bactericidal action against *Bacillus cereus* and *Escherichia coli*. *Biochemical Journal*, *391*, 167–175.

Wang, X., Du, Y., & Liu, H. (2004). Preparation, characterization and antimicrobial activity of chitosan–Zn complex. *Carbohydrate Polymers*, *56*, 21–26.

Wu, H., Zhang, J., Xiao, B., Zan, X. L., Gao, J., & Wan, Y. (2011). N-(2-hydroxypropyl)-3-trimethylammonium chitosan-poly(ε-caprolactone) copolymers and their antibacterial activity. *Carbohydrate Polymers*, *83*, 824–830.

Xing, K., Chen, X. G., Kong, M., Liu, C. S., Cha, D. S., & Park, H. J. (2009). Effect of oleoyl–chitosan nanoparticles as a novel antibacterial dispersion system on viability, membrane permeability and cell morphology of *Escherichia coli* and *Staphylococcus aureus*. *Carbohydrate Polymers*, *76*, 17–22.