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Antibacterial characteristics and activity of acid-soluble chitosan

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Abstract

The antibacterial activity of chitosan was investigated by assessing the mortality rates of *Escherichia coli* and *Staphylococcus aureus* based on the extent of damaged or missing cell walls and the degree of leakage of enzymes and nucleotides from different cellular locations. Chitosan was found to react with both the cell wall and the cell membrane, but not simultaneously, indicating that the inactivation of *E. coli* by chitosan occurs via a two-step sequential mechanism: an initial separation of the cell wall from its cell membrane, followed by destruction of the cell membrane. The similarity between the antibacterial profiles and patterns of chitosan and those of two control substances, polymyxin and EDTA, verified this mechanism. The antibacterial activity of chitosan could be altered by blocking the amino functionality through coupling of the chitosan to active agarose derivatives. These results verify the status of chitosan as a natural bactericide.

Keywords: Chitosan; Antibacterial action; Bactericide; Antibacterial activity

1. Introduction

Chitin and its deacetylated form chitosan are two of the most common polymers found in nature, with chitin second only to cellulose in terms of natural abundance (Sanford and Hutchings, 1987). Both chitin and chitosan are present in the shells of crustaceans and insects and in certain other organisms, including many fungi, algae, and yeast. Structurally, chitin and chitosan are biopolymers composed of *N*-acetylated glucosamine and glucosamine units linked by β(1–4) glycosidic bonds. Both polymers have been shown to have various promising biological activities, including antimicrobial, antitumor, and hemostatic activity, and the acceleration of wound-healing (Kurita, 1998), with chitosan, as a result of its deacetylation, having the additional properties of water solubility, biodegradability, and biocompatibility. Consequently, chitinous products have received considerable attention in recent years and have found applications in such industrial sectors as food production, pharmaceutical, agriculture, and biotechnology. Chitosan is often used as a chelating agent during the recovery of heavy metals from industrial wastewaters, and it can also react readily with negatively charged substances commonly found in the wastewater of food processing plants, such as proteins, polysaccharides, fatty acids, bile acids, and phospholipids (Jun et al., 1994; Muzzarelli, 1996; Bassi et al., 1999).

The natural antibacterial and/or antifungal characteristics of chitosan and its derivatives (Papineau et al., 1991; El-Ghaouth et al., 1992a; Sudarshan et al., 1992; Kim et al., 1997; Chung et al., 2003) have resulted in their use in commercial disinfectants. Chitosan has several advantages over other types of disinfectants in that it possesses a higher antibacterial activity, a broader spectrum of activity and a lower toxicity for mammalian cells (Liu et al., 2001). Applied in a spray form to fresh fruit, such as tomatoes and strawberries, chitosan is often used to extend storage life (El-Ghaouth et al., 1992a,b). It is also used as a food

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preservative (Shahidi et al., 1999). Both chitin and chitosan have been shown to activate the defense system of a host and prevent the invasion of pathogens (Sudarshan et al., 1992). Generally, chitosan has a higher antifungal activity than chitin, but it is less effective against fungi with a chitin or chitosan component in their cell walls (Allan and Hardwiger, 1979). Sudarshan et al. (1992) found that chitosan exhibited a differential antibacterial activity that manifested itself, in order of decreasing effectiveness, as *Enterobacter aerogenes* > *Salmonellas typhimurium* > *Staphylococcus aureus* > *Escherichia coli*. Wang (1992) and Chen et al. (2002), however, found a different inhibition order. To explain these apparently conflicting results, two mechanisms have been proposed to explain chitosan's antibacterial activity (Sudarshan et al., 1992). In the first, positively charged chitosan reacts with negatively charged molecules at the cell surface, thereby altering cell permeability and resulting in material being stopped from entering the cell and/or material being leaked from the cell. Several studies have illustrated the phenomenon by measuring leakage of glucose and lactate dehydrogenase from *E. coli* cells, or using hydrophobic membrane probes, chemical and electrophoretic analysis, and TEM technique for some Gram-negative bacteria (Tsai and Su, 1999; Helander et al., 2001). The second mechanism involves the binding of chitosan with DNA to inhibit RNA synthesis. It is very possible that the antibacterial actions of chitosan are a combination of both. The first mechanism is currently presumed to be due to outer membrane damage, but the results of recent experiments in the authors' laboratory suggest that the mechanism may not be that simple because it involves changes in the hydrophilicity and charge density of the cell surface as well as changes in the characteristics of chitosan adsorption to the cell wall (Chung et al., 2004).

The objectives of the research reported here were, therefore, to examine the antibacterial action of chitosan in detail and to demonstrate the antibacterial action of chitosan irrefutably with a focus on verifying the first mechanism.

2. Methods

2.1. Materials

Chitosan (source: shrimp; MW = 3×10^4), with an average degree of deacetylation (DD) of 95% or 75%, was purchased from Shin Dar Biotechnology Company (Taipei, Taiwan). Strains of *E. coli* (ATCC 25922) and *S. aureus* (ATCC 27853) were obtained from the American Type Culture Collection (ATCC). Fresh inoculants of these strains for use in assessing the antibacterial mechanism of chitosan were cultured on nutrient agar at 37 °C for 24 h. Nutrient broth was used to cultivate the experimental strains. Both the growth and test media were obtained from Difco Company. All chemicals were purchased from Sigma Chemical (St. Louis, MO) unless otherwise indicated.

2.2. Enzyme and nucleotide assays

Chitosan with 95% or 75% DD was dissolved in 0.2 M acetic acid and added to a 0.85% (w/w) NaCl solution containing *E. coli* and *S. aureus* at 10^7 CFU/ml at 37 °C. The pH of the suspension was adjusted to 5.6 by 0.1 N NaOH or HCl, and the final concentrations of chitosan and acetic acid were 2500 mg/l and 0.04 M, respectively. A 0.2-ml aliquot of the cell suspension was removed at hourly intervals and added to each of the reaction mixtures listed below. The amounts of enzyme and nucleotides released from the cells were determined according to standard procedures (see below).

Alkaline phosphatase was determined using the method reported 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 on a Beckman spectrophotometer. A unit of released alkaline phosphatase was defined as the amount of enzyme that produced 1 μM of *p*-nitrophenol-equivalent in 1 min at 28 °C. Glucose-6-phosphate (G-6-P) dehydrogenase was assayed in a solution (total volume: 1 ml) containing 0.05 M Tris–HCl buffer (pH 8), 0.01 M $CaCl_2$, 1.0 μmol glucose-6-phosphate, and 0.4 μmol TPN (Malamy and Horecker, 1964). The reduction of TPN at 28 °C was followed at 340 nm. A unit of released G-6-P dehydrogenase was defined as the amount of enzyme that reduced 1 μM of TPN-equivalent in 1 min at 28 °C. To prevent the interference of other molecules – proteins, for example – the cell solution was pretreated with a pure phenol/chloroform mixture and then centrifuged at 8000*g* for 5 min. The amount of nucleotides was then determined in the suspended liquid that was drawn off by measuring absorbance (OD) at 260 nm using a Beckman spectrophotometer (Neu and Heppel, 1964).

2.3. Comparison with various chemicals

E. coli cells were initially cultured in nutrient broth at 37 °C for 8 h. For harvesting of the cells, the suspension culture was first centrifuged (8000*g* for 5 min) and the supernatant removed; the pellet was then washed with the original volume of 0.01 M Tris–HCl buffer (pH 7.4), recentrifuged (8000*g* for 5 min) and the supernatant removed once again; the pellet was then resuspended in a solution of 0.85% (w/w) NaCl for subsequent experiments. Different concentrations of polymyxin B (50, 100, or 200 mg/l), ethylenediaminetetraacetic acid (EDTA) (500, 1000, or 2000 mg/l), and chitosan (95% DD; 1500, 2000, or 2500 mg/l) were added separately to different NaCl cell suspensions. The final mixture contained *E. coli* at 10^7 CFU/ml, and the pH of the different mixtures was adjusted (chitosan: pH 5.6; polymyxin B or EDTA: pH 7.2) using 0.1 N HCl. Temperature was maintained at 37 °C. Aliquots of 4 ml were removed from each reactive mixture at hourly

intervals to assay nucleotide release. The amounts of nucleotides in the samples were determined by measuring absorbance at 260 nm in a Beckman spectrophotometer.

2.4. Identification of the active functional group of chitosan

Chitosan with 95% or 75% DD was dissolved in 0.2 M HCl, and the pH of the solution was adjusted to 5.6. One gram of Affi-Gel 15 (Bio-Rad, Hercules, CA) was slowly added to different concentrations of chitosan solution (3000–5000 mg/l), resulting in specific concentrations of a supposed chitosan–agarose complex. Affi-Gel 15, which is derived from agarose beads, has an active carboxy-*N*-hydroxysuccinimide group that enables it to react with the amino group of chitosan by means of an amide bond. The mixture was gently stirred for 3 h at 4 °C to facilitate preliminary bead formation; these beads were then washed successively with 1 M NaCl, distilled water, and pure ethanol by a repeated dumping–washing process. A stable chitosan–agarose complex was produced by the freeze-dried method, and the concentration of chitosan in the chitosan–agarose complex was estimated by the Kjeldahl method (Lin, 1995). Agarose, chitosan, or the chitosan–agarose complex was added to a 0.85% (w/w) NaCl solution, pH 5.6, containing 10^7 CFU/ml of *E. coli* to a final concentration of agarose, chitosan, or chitosan in the chitosan–agarose complex of 2500 mg/l. Temperature was maintained at 37 °C in an orbital shaker incubator. At the same time, 1 g of Affi-gel 15 was mixed with various concentrations of chitosan solution (500, 1500, 2500, and 3500 mg/l) to generate a range of chitosan–agarose derivatives that were used in a separate experiment. Aliquots (0.1 ml) of the mixture were sampled hourly, inoculated onto nutrient agar, and incubated at 37 °C for 24 h, at which time the cell number was calculated by the plate count method.

2.5. Protoplast and spheroplast formation

Cultures from *S. aureus* and *E. coli* were grown in the nutrient broth at 37 °C for 8 h. For harvesting of the *S. aureus* and *E. coli* cells, the cell suspensions were centrifuged (8000*g* for 5 min) and the supernatant removed; the pellet was then washed with the original volume of TE buffer (10 mM Tris–HCl, 1 mM EDTA, pH 7.5), re-centrifuged (8000*g* for 5 min) and the supernatant removed once again; the pellet was then resuspended in a solution of TE buffer. A lysozyme suspension (10 mg/l) was then added to the *E. coli* buffer solution and incubated for 6 min at 30 °C. For *S. aureus*, a lysozyme suspension (100 mg/l) was added to the *S. aureus* buffer solution and incubated at 30 °C for at least 10 min. These suspensions were stirred gently at 100 rpm. Every 2 min, 0.5 ml of suspension was taken and checked by microscope until there was evidence of spherical protoplast or spheroplast formation (Malamy and Horecker, 1964).

2.6. Antibacterial tests of chitosan for intact cells, protoplasts, or spheroplasts

The intact cells, protoplasts, or spheroplasts were diluted to 10^7–10^8 CFU/ml in TE buffer and then mixed with chitosan (95% DD) to a final chitosan concentration of 2500 mg/l and pH of 7.5. The suspension was then incubated for 6 h at 37 °C. Control experiments (without the addition of chitosan) were also conducted. Each hour, 0.1 ml of the suspension was drawn off, used to inoculate nutrient agar, and incubated at 37 °C for 24 h, at which time the cell number was calculated by the plate count method. The antibacterial activity or removal efficiency of chitosan for intact cells, protoplasts, or spheroplasts was defined as:

$$\text{Removal efficiency } (\%) = \frac{(\text{initial cell number} - \text{cell number after treatment})}{\text{initialcellnumber}} \times 100\%$$

2.7. Transmission electron microscopy (TEM)

For the TEM study, intact cells, protoplasts, or spheroplasts of bacteria treated with chitosan (2500 mg/l) were drawn off after 2, 4, and 6 h of contact time and centrifuged at 8000*g*. The resulting pellets were resuspended in 0.1 M sodium phosphate buffer (pH 7.2, 25 °C) containing 4% glutaraldehyde solution to fix the cells. After a 4-h fixation period, the cells were washed twice with the same buffer and prepared for electron microscopy by post-fixation in 1% OsO_4 in 0.1 M sodium phosphate buffer (pH 7.2) for 6 h at 25 °C, two washes with the same buffer, dehydration in a graded series of ethanol, one wash with acetone, and embedding in Epon Lx-112. Thin sections were cut with a knife on a Reichert Ultracut ultramicrotome and double-stained with uranyl acetate and lead citrate. Transmission electron micrographs were taken using a JEM-100 CXII transmission electron microscope (JEOL, Japan).

2.8. Statistical analysis

All experiments were carried out in triplicate, and average values with standard deviation errors are reported. Mean separation and significance were analyzed using the SPSS software package (SPSS, Chicago, IL).

3. Results and discussion

3.1. Effect of chitosan on the leakage of enzymes and nucleotides from cells

The effects of chitosan on the leakage of cell inclusions from *S. aureus* and *E. coli* cells over an 8-h period are shown in Table 1 as units (or amounts) of average release at the steady state condition. The controls, which consisted

Table 1
Effect of chitosan on the leakage of enzymes and nucleotides from *Staphylococcus aureus* and *Escherichia coli*

	Staphylococcus aureus (ATCC 27853)			*Escherichia coli* (ATCC 25922)		
	Alkaline phosphatase (Units)[a]	G-6-P dehydrogenase (Units)[b]	Nucleotides (OD)	Alkaline phosphatase (Units)[a]	G-6-P dehydrogenase (Units)[b]	Nucleotides (OD)
DD 95%	180.2 ± 21.3	274.2 ± 22.5	0.91 ± 0.09	220.3 ± 17.5	405.1 ± 26.8	1.03 ± 0.14
DD 75%	95.5 ± 7.4	142.7 ± 4.6	0.80 ± 0.03	115.5 ± 8.6	173.9 ± 13.5	0.88 ± 0.06

Experimental conditions: concentration of chitosan, 2500 mg/l; concentration of bacterial cells, 10^7 CFU/ml; initial pH, 5.6; reactive temperature, 37 °C. Data are presented as amounts or units of release at the steady state for 8-h periods ± one standard deviation from the mean ($n = 3$).

[a] The unit of alkaline phosphatase release was defined as the amount of enzyme that produced 1 μM of *p*-nitrophenol-equivalent in 1 min at 28 °C.

[b] The unit of G-6-P dehydrogenase release was defined as the amount of enzyme that reduced 1 μM of TPN-equivalent in 1 min at 28 °C.

of bacteria in a 0.04 M acetic acid solution or in a 0.85% NaCl solution, showed zero units (or OD) of enzymes and nucleotides released. The addition of chitosan with a high degree of deacetylation resulted in a high leakage of enzymes from both *S. aureus* and *E. coli* cells equally. With increasing contact time between the chitosan and the bacterial cells of all strains tested, the leakage of enzymes from the cells increased gradually, reaching a plateau in 2–5 h (Fig. 1a and b). The leakage of nucleotides exhibited a smoothly ascending curve during the 8-h period (Fig. 1c). The leakage of alkaline phosphatase reached a steady state (*i.e.* a plateau) by 2 h, while glucose-6-phosphate dehydrogenase required 5 h. The addition of chitosan to the bacterial suspension seemed to have a stronger impact on the Gram-negative *E. coli* than on the Gram-positive *S. aureus* in terms of the leakage of enzymes. Malamy and Horecker (1964) reported that alkaline phosphatase is an extracellular enzyme, while glucose-6-phosphate dehydrogenase is found in the cell membrane and nucleotides are located in the cytoplasm. The (extra)cellular locations of these molecules clearly provide the answer as to why alkaline phosphatase was the first (fastest) molecule to be released into the medium, followed by glucose-6-phosphate dehydrogenase and finally by nucleotides (slowest).

The experimental results also reveal that the antibacterial action of chitosan not only involves a reaction with the cell wall of the bacteria but that it also may affect the structure of the phospholipid bilayer in the cell membrane, thereby changing the permeability of the cell membrane, resulting in the release of some of the cellular components. This action was further enhanced when chitosan with a high degree of deacetylation was used (Fig. 1).

3.2. Antibacterial action of chitosan in comparison with that of an antibiotic and a chemical chelating agent

To gain a better understanding of the mechanism by which chitosan functions as a bactericide, the cells were subjected to polymyxin B, a known antibiotic, and EDTA, a chemical chelating agent, and the results compared to the antibacterial action of chitosan. Based on published reports, the cationic antibiotic polymyxin B reacts with the anionic phosphate group of phospholipids in the cell membrane, thereby destroying the cell membrane structure and affecting its permeability (Newton, 1956). EDTA, on the other hand, destroys the structure of the cell wall by chelating with the Ca^{2+} or Mg^{2+} present in the cell wall (Asbell and Eagon, 1966).

Fig. 2 shows that the leakage of nucleotides from the bacterial cells increases with increasing contact time and concentrations of polymyxin B, EDTA, and chitosan. The antibacterial profiles, based on the OD measurements, were linear for polymyxin B and rose in a smooth curve for EDTA at all three concentrations tested. However, the antibacterial curves for chitosan exhibited two phases: a smooth increase followed by a linear increase. Assuming that the cationic polymyxin B reacted directly with the bacterial cell membranes, this result shows that the release of nucleotides was almost directly in proportion to the contact time. The bacteriostatic action of EDTA, however, destroys the complex structure of the cell wall (especially in Gram-negative bacteria) (Helander et al., 2001), with the result that the release of nucleotides from bacterial cells in contact with EDTA would be a smooth curve due to the presence of the nucleotides in the cytoplasm. Chitosan chelates metal ions easily (Bassi et al., 1999); as such, the lower rate of nucleotide leakage from bacterial cells cultured with chitosan resembled that caused by EDTA during the first phase (from 1 to 3 h) of the incubation. With the eventual destruction of the cell-wall structure, the amount of leakage during the second phase (from 4 to 8 h) increased further and was again directly proportional to the contact time. The antibacterial action of chitosan during this phase may be compared to the behavior of polymyxin B because cationic polyelectrolyte chitosan would function in a similar manner to this antibiotic due to the existence of the NH_3^+ functional group. Interestingly, a transition stage (from 3 to 4 h) can also be seen in Fig. 2. One proposal is that the cell membranes were destroyed at this time (data was replicated three times). Based on these results, it would appear that the antibacterial action of chitosan has a double mechanism, affecting both the cell wall and the membrane. To date, this finding has not been reported in the literature. To confirm the effect of the solvent (0.01 M Tris–HCl buffer or 0.04 M acetic acid) on the leakage of nucleotides, the controls were also examined: there was

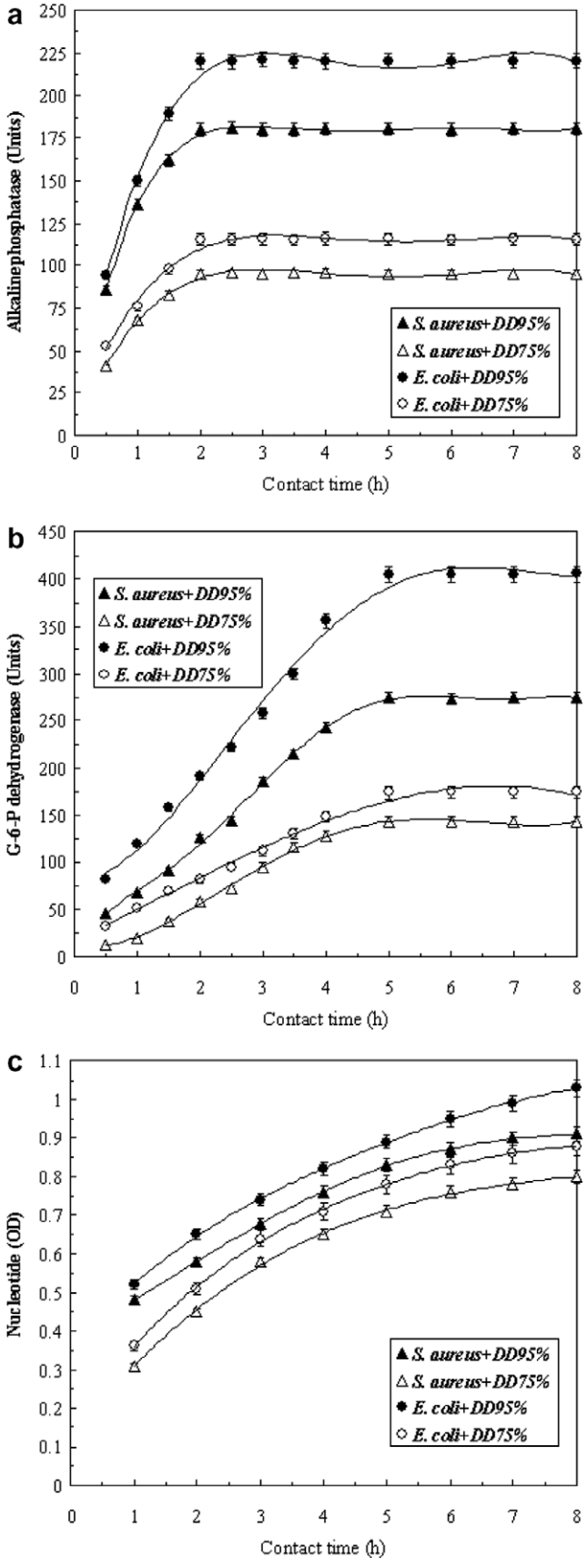

Fig. 1. The effects of chitosan (2500 mg/l) with 95% or 75% DD on the leakage of cell inclusions from *Staphylococcus aureus* and *Escherichia coli* cells following different contact times.

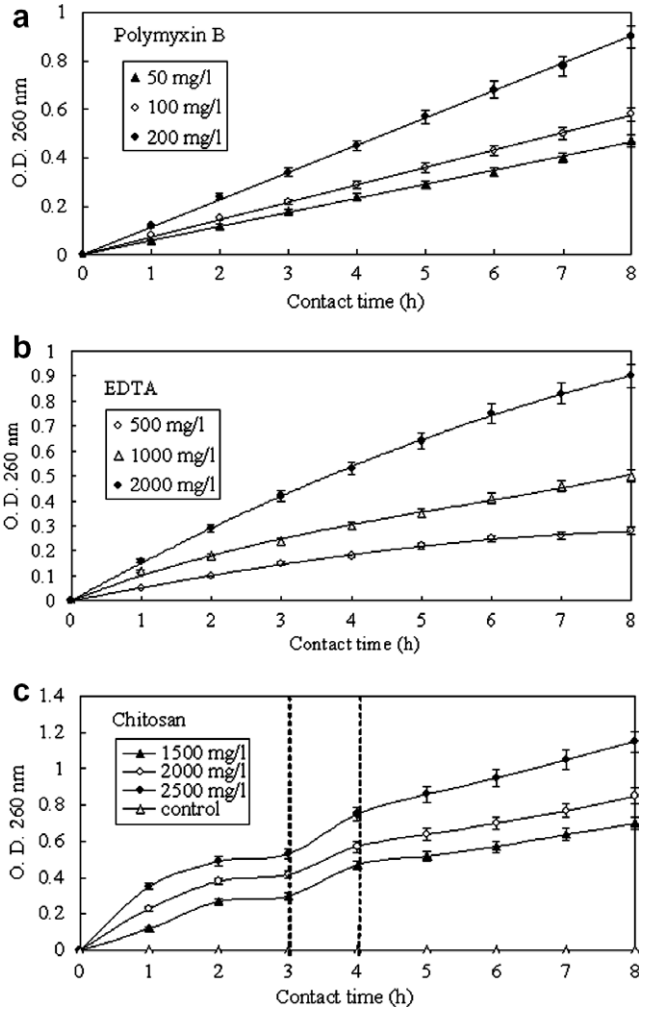

Fig. 2. Effects of different chemicals [polymyxin B (a), EDTA (b) and chitosan (c)] on the leakage of nucleotides from *Escherichia coli*. The initial cell number was 10^7 CFU/ml, and the temperature was maintained at 37 °C. The pH values of mixtures containing polymyxin B, EDTA, or chitosan were 7.2, 7.2, and 5.6, respectively. The results are the means of triplicate tests, and error bars indicate the standard deviations.

no leakage of nucleotides from bacterial cells incubated in 0.01 M Tris–HCl buffer (data not shown) and only very limited leakage from cells incubated in 0.04 M acetic acid (Fig. 2c).

3.3. Identification of the active functional group of chitosan

The antibacterial activities of the chitosan–agarose complex, chitosan, and agarose derivative against *E. coli* and the effects of various ratios of chitosan and agarose derivative on the survival of *E. coli* are shown in Fig. 3. Fig. 3a clearly shows that the solvent (control: 0.04 M acetic acid) and agarose derivative alone had no significant effect on the survival of *E. coli*. Chitosan with 95% DD had higher antibacterial activity than chitosan with 75% DD, and their antibacterial activities increased with contact time. After 6 h of incubation, the removal efficiencies of chitosan with 95% DD and 75% DD were 99.999% and 99.984%,

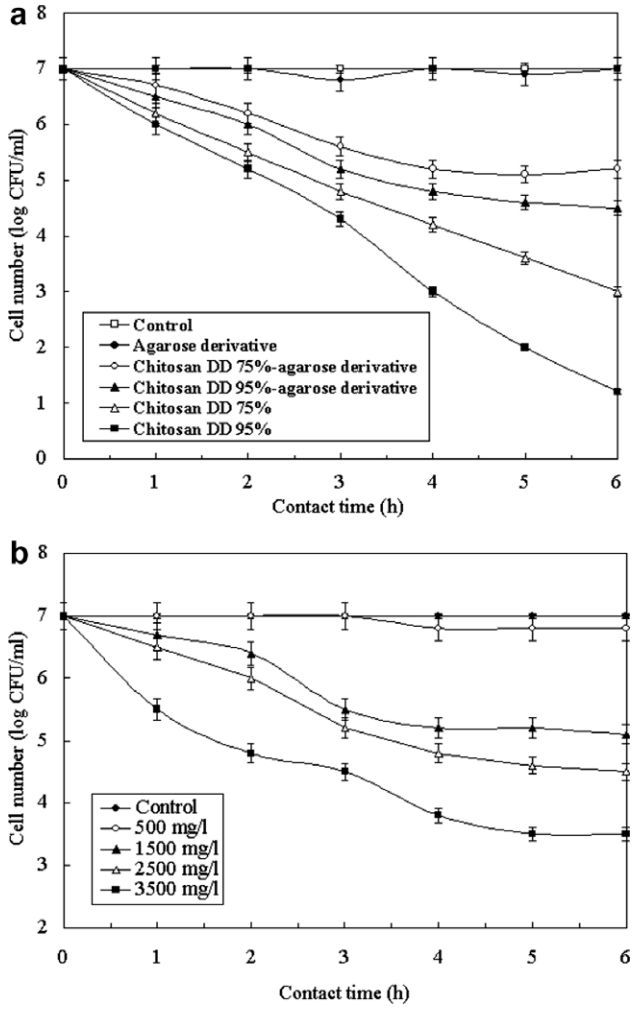

Fig. 3. Antibacterial activities of the chitosan–agarose derivative and chitosan against *Escherichia coli* (a) and the effects of different mixed concentrations of chitosan (95% DD) with agarose derivatives on the survival of *Escherichia coli* (b). The final concentration of agarose derivative, chitosan, or chitosan in the chitosan–agarose complex was 2500 mg/l in (a). The results are the means of triplicate tests, and error bars indicate the standard deviations.

respectively. Conversely, the removal efficiencies for the chitosan–agarose complex with 95% DD and 75% DD were 99.602% and 96.838%, respectively. The time required to reach a steady state with the chitosan–agarose complex was approximately 4 h. The lower antibacterial activity of the chitosan–agarose complex is clearly evident in Fig. 3a. Two possible explanations for this lower activity are that the antimicrobial functional group of chitosan in the chitosan–agarose complex is shielded or that the chitosan–agarose complex hinders its action sterically. Since the chitosan with the amino group may react with the active carboxy-N-hydroxysuccinimide group of the agarose derivative Affi-Gel 15 by amide bonding, the loss of free amino groups on the chitosan molecule may result in low antibacterial activity (Sudarshan et al., 1992; Wang, 1992) based on the generally accepted assumption that the antimicrobial functional group of chitosan is the free amino group.

The antibacterial activity of the chitosan + agarose derivative against *E. coli* at different concentration ratios is shown in Fig. 3b. The various combinations of chitosan + agarose derivative were produced by reacting the quantitative agarose derivative with different concentrations of chitosan. Fig. 3b clearly shows that the antibacterial activity of the chitosan + agarose derivative increased with higher concentrations of chitosan. However, *E. coli* cells cultured with 500 mg/l chitosan–agarose derivative were almost completely unaffected and essentially showed a similar result to the control (0.04 M acetic acid; $p > 0.05$). While this result initially appears to be inconsistent with the known antibacterial activity of chitosan, it clearly reflects the fact that in this case the quantitative agarose derivatives had reacted with all, or nearly all of the amino groups on the chitosan molecules, thereby essentially inactivating the antibacterial functional group. When 500 mg/l chitosan was incubated with *E. coli* cells, an approximately 98.415% removal of *E. coli* cells was achieved (data not shown).

3.4. Antibacterial tests of chitosan for intact cells, protoplasts, or spheroplasts

The experimental results provide tangible evidence in support of the hypothesis that the amino group on chitosan is a source of antibacterial activity. Based on the observation that once the structure of the cell wall was destroyed, the amount of leakage increased at a faster rate, work focused on the role of the cell wall in the antibacterial mechanism of chitosan with the aim of obtaining a fuller understanding of this mechanism.

Fig. 4 shows the antibacterial activity of chitosan against intact cells and cells treated with lysozyme prior to being incubated with chitosan. The protoplast or spheroplast was much more sensitive to the environment than the intact cell when incubated in the absence of chitosan. The addition of chitosan to the suspension culture of *S. aureus* protoplasts or *E. coli* spheroplasts resulted in more cell deaths than when intact cells were used. The antibacterial curves of chitosan against *S. aureus* protoplasts and *E. coli* spheroplasts in Fig. 4 can be seen to fall sharply in comparison to those with intact cells. In addition, the removal ratios of chitosan for *S. aureus* protoplasts and *E. coli* spheroplasts were significantly higher than those for intact cells during the first 3–4 h of contact time. These results indicate strongly that the cell wall of the bacterial cells acts as an important protective barrier against chitosan which, once destroyed, would lead to a physiological situation in which the antibacterial activity of chitosan would be favored.

The intact bacterial cells, protoplasts, and spheroplasts treated with chitosan were examined by TEM after 2, 4, and 6 h of contact time. The TEM images revealed that the bacterial cell membrane gradually detached from the cell walls of the intact cells of both *E. coli* (Fig. 5b) and *S. aureus*, indicating that the Ca^{2+} and Mg^{2+} drawn from the cell wall by chitosan (Muzzarelli and Tanfani, 1982;

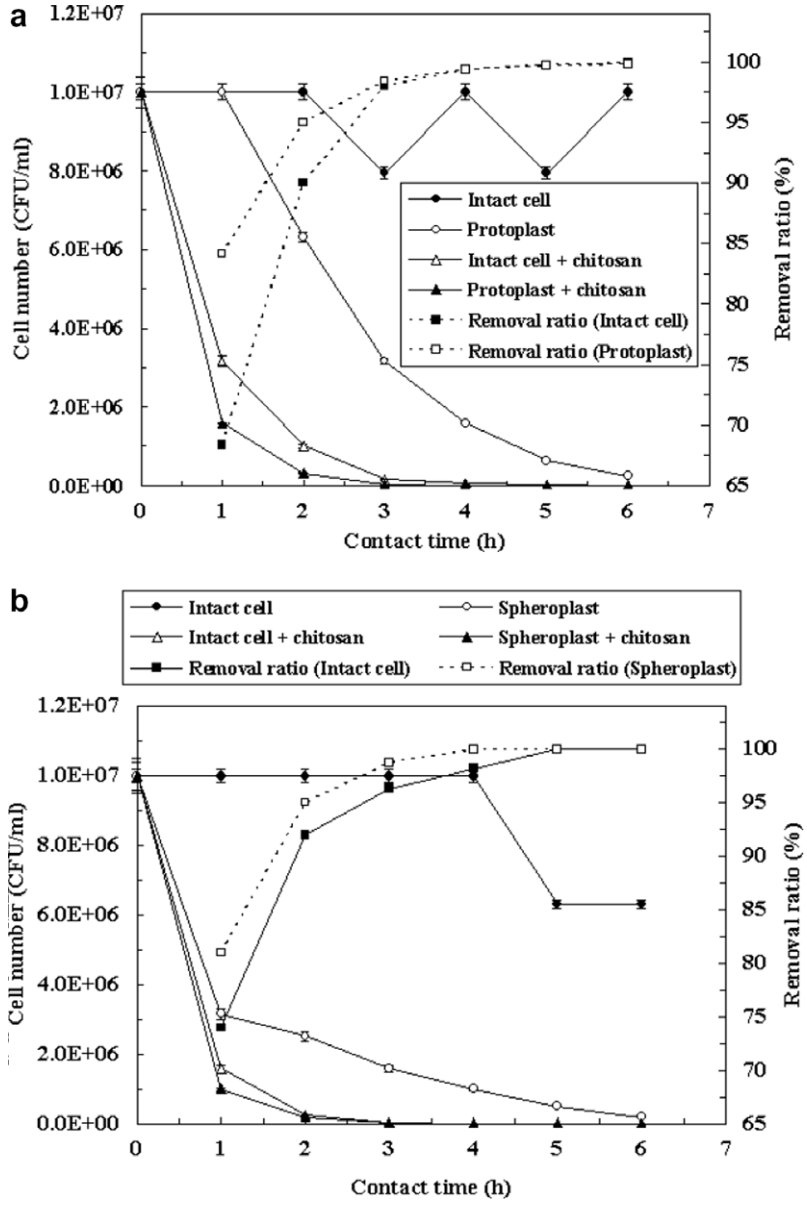

Fig. 4. Antibacterial activities of chitosan against intact cells and protoplasts of *Staphylococcus aureus* (a) and against intact cells and spheroplasts of *Escherichia coli* (b). Experimental conditions: 2500 mg/l of chitosan with 95% DD; initial pH, 7.5. The results are the means of triplicate tests, and the error bars indicate the standard deviations.

Ramachandran Nair and Madhauan, 1982; Rhazi et al., 2002; Chung et al., 2003) had destroyed the integrity of the cell wall. The images also showed that the cytoplasm of intact cells, protoplasts, and spheroplasts had broken down and that the contents of the cells, protoplasts, and spheroplasts gradually reduced in volume (Fig. 5c and d). The extent of this breakage was more extensive in the protoplast than in the spheroplast (data not shown).

4. Conclusions

The results reported here demonstrate that chitosan can destroy the cell structure of *E. coli* and *S. aureus* cells, resulting in the leakage of enzymes and nucleotides from different cell locations. The inactivation of *E. coli* by chitosan occurred through a two-phase sequential mechanism, which was subsequently verified by comparison with the antibacterial profiles and patterns of other chemicals. The amino group (NH_3^+) as the active functional group was found to be essential to the antibacterial activity of chitosan. The survival rates of intact cells, protoplasts and spheroplasts affected by chitosan indicate the importance of cell walls in the action of chitosan against bacteria. These data provide additional evidence in support of chitosan being regarded as a natural bactericide.

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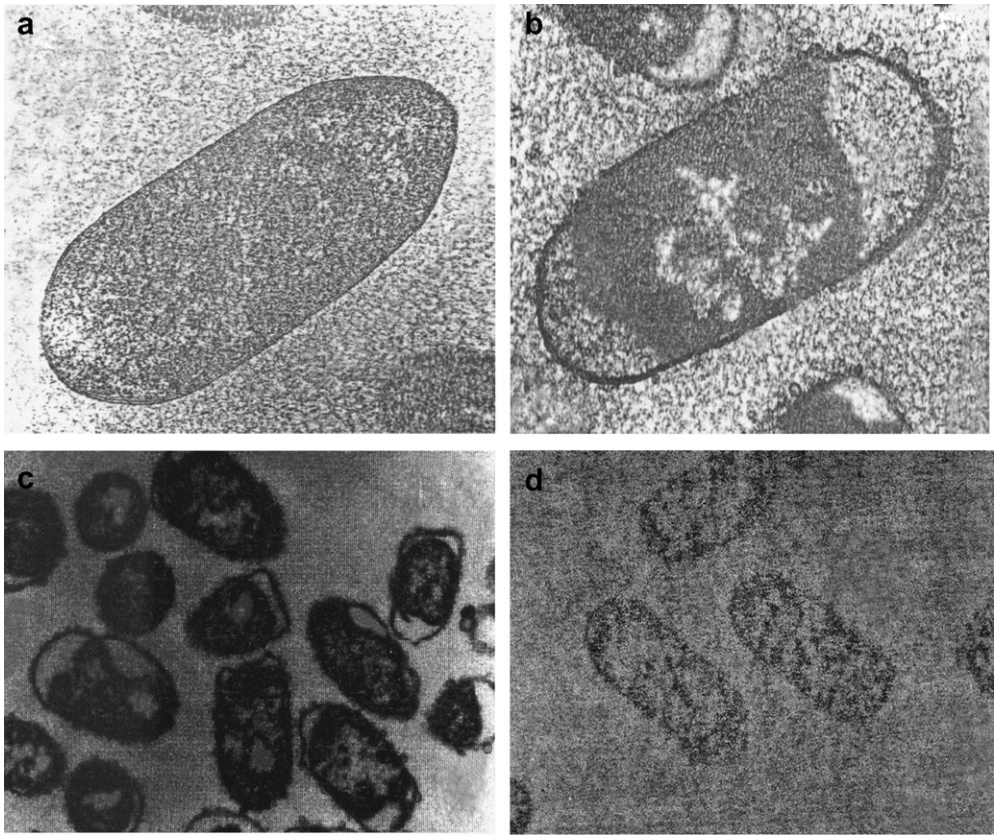

Fig. 5. TEM micrographs of intact cells of *Escherichia coli* when *Escherichia coli* was exposed to chitosan (2500 mg/l, 95% DD) after 0 h (a), 2 h (b), 4 h (c), 6 h (d), respectively.

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