Relationship between antibacterial activity of chitosan and surface characteristics of cell wall

Ying-chien CHUNG[1], Ya-ping SU[2,7], Chiing-chang CHEN[3], Guang JIA[4], Huey-lan WANG[5], J C Gaston WU[2], Jaung-geng LIN[6]

[1]*Department of Industrial Engineering and Management, China Institute of Technology, Taipei 245, China;*
[2]*Department of Chemistry, National Taiwan Normal University, Taipei 116, China;*
[3]*Department of General Studies, National Taichung Nursing College, Taichung 403, China;*
[4]*Department of Occupational and Environmental Health Sciences, School of Public Health, Peking University, Beijing 100083, China;*
[5]*Basic Medical Science Education Center, Fooyin University, Kaohsiung 831, China;*
[6]*Institute of Chinese Medical Science, China Medical University, Taichung 404, China*

KEY WORDS anti-bacterial agents; chitosan; cell wall

ABSTRACT

AIM: Five representative waterborne pathogens were used to illustrate the relationship between chitosan's antibacterial activity and the surface characteristics of the bacterial cell wall. **METHODS:** Chitosan was prepared with averaged 75 % or 95 % deacetylated degree to examine its antibacterial activity against waterborne pathogens. Fresh microbial inoculants for the antibacterial assessment were prepared on nutrient agar at 37 ºC for 24 h. The evaluation items of antibacterial mechanism included hydrophilicity and negative charge analysis of cell surface, and adsorptive characteristics of chitosan to bacterial cell. All the experiments were applied in triplicate tests at least. **RESULTS:** Although cell wall hydrophilicity was similar among Gram-negative bacteria, the distribution of negative charge on their cell surfaces was quite different. More negatively charged cell surfaces had a greater interaction with chitosan, a phenomenon further confirmed by transmission electron micrography (TEM). **CONCLUSION:** Results showed the hydrophilicity in Gram-negative bacteria was much higher than in Gram-positive ones. The correlation coefficient 0.988 between the amount of absorbed chitosan and its inhibition efficiency indicated a close relationship.

INTRODUCTION

Chitosan, β-(1-4) linked 2-amino-2-deoxy-*D*-glucose, is a natural biopolymer on earth after cellulose and deacetylated from chitin[1]. It is the main structural component of the cuticles of crustaceans, insects and molluscs and the cell walls of certain fungus and has been estimated that is produced in nature at a level of up to 1×10^9-1×10^{10} tones per year[2]. Chitosan is a natural, positively charged polysaccharide with a pK_a equal to 6.3-7[3] and it has a potential application in several areas, including food[4], pharmaceutical[5], biotechnology[6], and environment[7]. It exhibits various promising biological activities, including antimicrobial activity, antitumor activity, hemostatic activity, and acceleration of wound healing[5,8]. Chitosan is biodegradable and biocompatible[5]. Hence, extensive research has been conducted to explore its potential applications in vari-

[7] Corrspondence to Ya-ping SU.
Phn 886-2-2935-0749, ext 544 or 546. Fax 886-3-5228-451.
E-mail su451@ms37.hinet.net
Received 2003-09-01 Accepted 2004-01-30

ous industries.

Recently, research has shifted and focused on the possibility of developing chitosan as a natural disinfectant[9]. It can also be applied to extend the storage life of fresh fruit[10,11] and other foods[4]. Much of the interest in the antimicrobial properties of chitosan has focused on the possibility of plant protection[12]. Chen *et al* applied chitosan as a natural disinfectant against waterborne pathogens and proved it to be promising[13].

EI-Ghaouth *et al* have proposed possible antibacterial actions of chitosan and its derivatives[11]. They asserted that chitosan reacted with the cell surface, altered cell permeability, and further prevented the entry of material or caused the leakage of material. However, no evidence has been provided to demonstrate the relationship between the antibacterial activity of chitosan and the surface characteristics of the bacterial cell wall. In order to illustrate this relationship, we used five waterborne pathogens, including three Gram-positive and two Gram-negative bacteria, to analyze the hydrophilicity, the negative charge of the cell, and the adsorption amount of chitosan to the cell surface.

MATERIALS AND METHODS

Materials Chitosan, prepared with averaged 75 % or 95 % deacetylated degree (DD), was extracted from shrimp and purchased from Shin Dar Biotechnology Company (Taipei, China). Strains of waterborne pathogens, *Pseudomonas aeruginosa* (ATCC 27853), *Salmonella typhimurium* (ATCC 27198), *Escherichia coli* (ATCC 25922), *Staphylococcus aureus* (ATCC 27853), and *Streptococcus faecalis* (ATCC 4200) were obtained from the American Type Culture Collection (ATCC). Fresh inoculants for the experimental assessment were prepared on nutrient agar at 37 ºC for 24 h. *S faecalis* was cultivated in brain heart infusion nutrient and others were cultivated in nutrient broth. All growth media were obtained from Difco Company and chemicals were purchased from Sigma Chemical Company unless otherwise indicated.

Hydrophilicity analysis of cell surface Cell suspension (1×10^7 CFU/mL) was harvested from the cultivated broth after a two-day cultivation and added to 5 mL of a two-phase mixture, with various ratios of hexane and water. The solution was mixed for 3 min and allowed to settle for 5 min. The absorption of the lower part of the solution (water phase) was measured at 660 nm in the Beckman spectrophotometer. The hydrophilicity was expressed as determined value divided by control value[14].

Negative charge analysis of cell surface Anion exchange resin, Dowex-2, was washed five times with deionized water and balanced with 0.1 mol/L HCl in the burette for 6 h. Five milliliters of cell suspension (1×10^7 CFU/mL) were sequentially added to the burette and mixed completely. After Dowex-2 settled to the bottom of the burette, the absorption of the upper part of the solution (water phase) was measured at 660 nm in the Beckman spectrophotometer. The relative cell density (RCD) was expressed as determined value divided by control value[15], and relative absorbed ratio was equal to 100 %-RCD.

Adsorptive characteristics of chitosan to bacterial cell Chitosan (2500 ppm) with 75 % DD or 95 % DD was added to 100 mL cell suspension (1×10^7 CFU/mL). The mixture was adjusted to pH 4.0 or 5.0 with 0.1 mol/L HCl and then shaken at 30 r/min. Every hour, the upper solution was collected (after settling for 10 min) to analyze the residual chitosan. The adsorptive balance between cells and chitosan reached a steady state in about 3 h. The adsorptive capacity (amount) was expressed as an average during the 3- to 6-h experiment. Chitosan analysis was done according to the titration method presented by Tsuji and Kinoshita[16].

RESULTS

Hydrophilicity analysis of cell surface Fig 1 showed the hydrophilicity of the cell wall in the tested bacteria. Results indicated the residual amounts of Gram-positive strains *Staphylococcus aureus* and *Streptococcus faecalis* in the water phase were gradually decreased with the increasing volume of hydrophobic *n*-hexane. However, the residual amounts of Gram-negative bacteria *Pseudomonas aeruginosa, Salmonella typhimurium,* and *Escherichia coli* were indifferent to the addition of *n*-hexane. The hydrophilicity order was *Pseudomonas aeruginosa, Salmonella typhimurium, Escherichia coli, Staphylococcus aureus* and then *Streptococcus faecalis*. The difference among the Gram-negative bacteria was not significant ($P>0.05$).

Negative charge analysis of cell surface Relative cell density (RCD) was used to represent the residual amount of cells in the solution after adsorption on Dowex-2 resin. A lower RSD represented a higher negatively charged density distributed over the cell surface. Tab 1 showed *Staphylococcus aureus* had higher negatively charged density on its cell surface than *Streptococcus faecalis* did. As for Gram-negative bacteria,

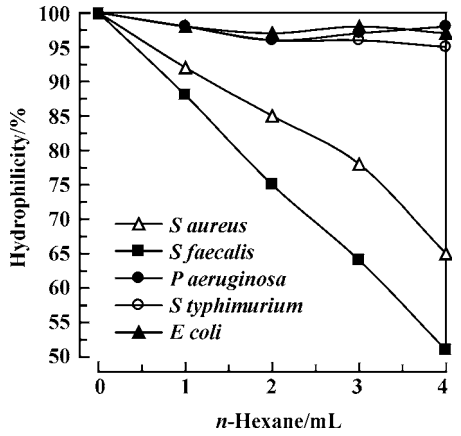

Fig 1. Hydrophilicity of bacterial cell in biphasic partition with different volumes of *n*-hexane.

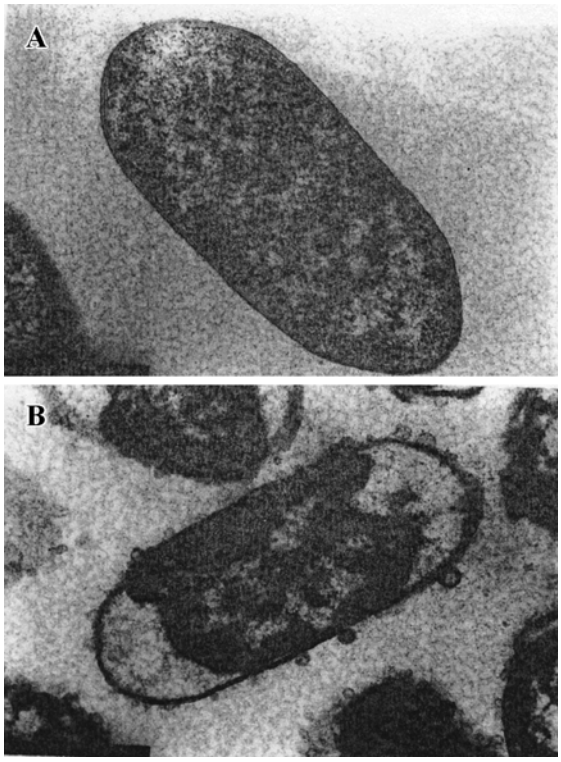

Fig 2. A) Control group *Escherichia coli* before adsorption of chitosan. B) TEM diagram of adsorption of chitosan on *E coli* after 4 h. (TEM×4000).

Pseudomonas aeruginosa carried the highest negative charge. *Samonella typhimurium* was second, and *Escherichia coli* was third. Besides, the negative charge on the cell surface of Gram-negative bacteria was higher than that on Gram-positive bacteria. The data of hydrophilicity, RCD, and inhibition capacity of chitosan against the tested bacteria (the data obtained from reference 13) were collected and treated by regression analysis. Fig 2 showed the correlation coefficient of hydrophilicity and inhibition efficiency of chitosan was 0.824, and that of relative adsorbed ratio (100%-RCD) and inhibition efficiency of chitosan was 0.942, respectively.

Adsorptive characteristics of bacterial cell to chitosan To further illustrate the effect of the negative charge of the cell surface on the antibacterial activity of chitosan, the amount of bactericide chitosan adsorbed to the different bacterial cells was determined and listed in Tab 2. It showed that more chitosan were adsorbed to Gram-negative than Gram-positive bacteria, and the sequence was *Pseudomonas aeruginosa*, *Salmonella typhimurium*, *Escherichia coli*, *Staphylococcus aureus*, and *Streptococcus faecalis.* Tab 2 also indicated the adsorbed amounts of chitosan were related to environmental pH values and degree of deacetylation of chitosan. Chitosan was adsorbed by bacterial cells more at pH 4.0 than at pH 5.0. In addition, chitosan with a higher degree of deacetylation would result in greater adsorbed amounts. The relationship between adsorbed amounts of chitosan by bacterial cell and the inhibition efficiency of chitosan (20 groups) was analyzed by the regression method. Fig 3 revealed the correlation coefficient between both is 0.925. This will increase to 0.988 if the data of 100 % inhibition efficiency (5

Tab 1. Residual percentage of bacterial cell in the solution after the adsorption on anion exchange resin Dowex-2.

Tested Bacteria	G (–)			G (+)	
	Pseudomonas aeruginosa	*Samonella typhimurium*	*Escherichia coli*	*Staphylococcus aureus*	*Streptococcus faecalis*
Relative cell density*	15 %	36 %	48 %	62 %	74 %

*Relative cell density =(cell density in the solution with Dowex-2 treatment/cell density in the solution without Dowex-2 treatment) ×100 %.

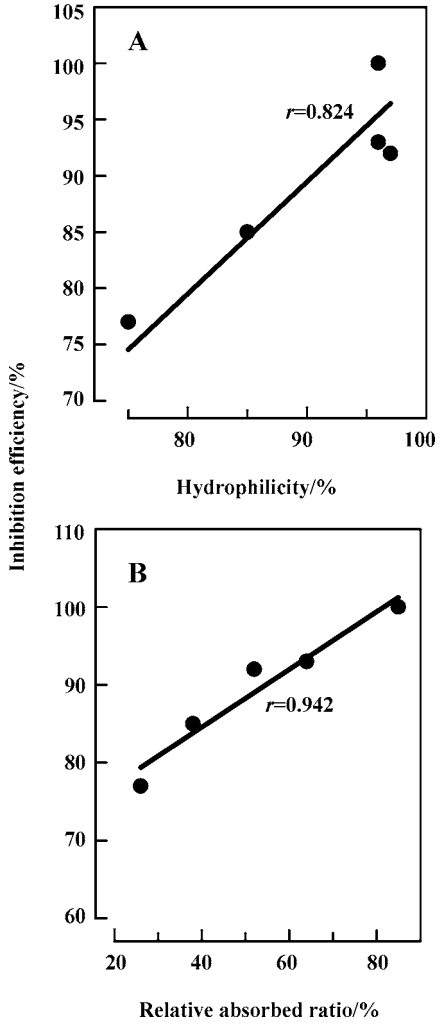

Fig 3. A) Relationships between hydrophilicity and inhibition efficiency of chitosan. B) Relationships between hydrophilicity and inhibition efficiency of chitosan and between relative adsorbed ratio (%) and inhibition efficiency of chitosan.

groups) are eliminated, because they are regarded as reaching adsorptive saturation. To clearly explain and prove the adsorption phenomenon, the TEM diagrams for adsorption of chitosan on *Escherichia coli* was presented in Fig 4.

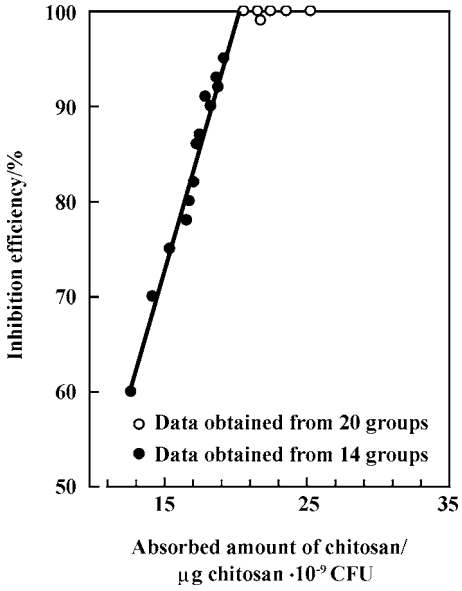

Fig 4. Relationship between absorbed amount of chitosan and inhibition efficiency of chitosan.

DISCUSSION

Chitosan or its derivatives have been proven more effective for Gram-negative bacteria than Gram-positive bacteria[13]. Although the antibacterial mechanism has been interpreted, little evidence has been provided to demonstrate the relationship between the antibacterial activity of chitosan and the surface characteristics

Tab 2. The absorbed amounts of chitosan with various deacetylated degree at pH 4 or pH 5 on different bacterial cells.

Condition	G (–) *Pseudomonas aeruginosa*	G (–) *Samonella typhimurium*	G (–) *Escherichia coli*	G (+) *Staphylococcus aureus*	G (+) *Streptococcus faecalis*
pH=5					
DD 95 %	22.5	20.6	18.7	17.5	16.8
DD 75 %	18.8	17.3	15.4	14.2	12.7
pH=4					
DD 95 %	25.3	23.6	21.8	18.3	17.9
DD 75 %	21.6	19.2	17.1	16.6	15.4

The unit of adsorbed chitosan was μg chitosan/1×10^9 CFU.

of cell wall. In this paper, the relationship was exposed by using a biphasic partition system and it provided a feasible direction for advanced study. Comparison with the inhibition degree of chitosan to tested bacteria[17], the antibacterial activity of chitosan seemed to be related with the hydrophilicity of cell wall. However, the relationship between the inhibition capacity of chitosan and hydrophilicity of cell wall for Gram-negative bacteria needed further evaluation.

As the hydrophilicity of the cell wall of Gram-negative bacteria could not fully explain the difference in the antibacterial activity of chitosan, the charge characteristics of cell surface were required to be determined. Results of negative charge analysis in Tab 1 indicated that the same tendency was observed in the hydrophilicity analysis (Fig 1). Hence, positively charged chitosan had higher antibacterial activity or inhibition activity in *Staphylococcus aureus* than *Streptococcus faecalis*[13]. Although hydrophilicity was similar among Gram-negative bacteria, the significant different negative charge distributed on the surface of cell wall of the tested strains. This order well fitted with the inhibition degree of chitosan against these tested bacteria[13,17]. This clearly explained why most Gram-negative bacteria were sensitive to chitosan and negative charge density on the cell surface apparently determined whether the bacteria were easily inhibited by chitosan or not.

Because the amount of adsorbed chitosan to the different bacterial cells is exactly the same order determined for the antibacterial activity of chitosan on these bacteria[13,17], the antibacterial activity of chitosan and the surface characteristics of the cell wall are closely related. More adsorbed chitosan would result in greater changes in the structure of the cell wall and in the permeability of the cell membrane. Both adverse effects results in the death of the bacteria. As chitosan easily carries more positively charged amino groups (NH_3^+) in more acidic solution and a higher degree of deacetylation, it would result in greater adsorbed amounts. The adsorption experiments clearly indicate the relationship between the antibacterial activity (inhibition efficiency) of chitosan and surface characteristics of the bacterial cell wall. Besides, TEM diagrams for adsorption of chitosan further verify the phenomenon.

ACKNOWLEDGEMENT We thank for assistance and advices on laboratory technology support by Kung-tung CHEN, Department of Genenal Education, Ming Hsin University of Science and Technology, Hsin-chu, Taiwan, China.

REFERENCES

1 Sanford PA, Hutchings GP. Genetic engineering, structure/property relations and application, New York: Elsevier; 1987.
2 Peter MG. Introductory remarks. Carbohydr Eur 1997; 19: 9-15.
3 Schulz PC, Rodriguez MS, Del Blanco LF, Pistonesi M, AgulloE. Emulsification properties of chitosan. Colloid Polym Sci 1998; 276: 1159-65.
4 Shahidi FJ, Arachchi KV, Jeon YJ. Food applications of chitin and chitosans. Trends Food Sci Technol 1999; 10: 37-51.
5 Kurita K. Chemistry and application of chitin and chitosan. Polym Degrad Stabil 1998; 59: 117-20.
6 Somashekar D, Joseph R. Chitosanases properties and application: a review. Bioresource Technol 1996; 55: 35-45.
7 Bassi R, Prasher SO, Simpson BK. Effects of organic acids on the adsorption of heavy metal ions by chitosan flakes. J Environ Sci Health A 1999; 34: 289-94.
8 Nam KS . Evaluation of the antimutagenic potential of chitosan oligosaccharide: Rec, Ames and Umu tests. Biotechnol Lett 2001; 23: 971-75.
9 Kim CH, Kim SY, Choi KS. Synthesis and antibacterial activity of water-soluble chitin derivatives. Polym Adv Technol 1997; 8: 319-25.
10 EI-Ghaouth A, Ponnampalam R, Castaigne F, Arul J. Chitosan coating to extent the storage life of tomatoes. Hortscience 1992; 27: 1016-18.
11 EI-Ghaouth A, Arul J, Asselin A, Benhamou N. Antifungal activity of chitosan on two post-harvest pathogen of strawberry fruits. Phytooath 1992; 82: 398-402.
12 Gooday GW. The ever-widening diversity of chitinases. Carbohydr Eur 1997; 19: 18-22.
13 Chen YM, Chung YC, Wang LW, Chen KT, Li SY. Antibacterial properties of chitosan in waterborne pathogen. J Environ Sci Health A 2002; 37: 1379-90.
14 Loosdrecht MM, Lyklema J, Norde W, Zehnder, AJB. The role of bacteria cell wall hydrophilicity in adhesion. Appl Environ Microbiol 1987; 53: 1893-98.
15 Zvaginstev G, Gusev VS. Concentration and separation of bacteria on Dowex anionite. Mikrobiologiya 1971; 40: 123-7.
16 Tsuji A, Kinoshita T. Analytical chemical studies on amino sugar. Chem Pharm Bull 1969; 17: 1505-12.
17 Wang GH. Inhibition and inactivation of five species of foodborne pathogens by chitosan. J Food Protect 1992; 55: 916-25.