Available online at www.sciencedirect.com
ScienceDirect
Carbohydrate Polymers 67 (2007) 66–72
Carbohydrate Polymers
www.elsevier.com/locate/carbpol

# Synthesis, characterization and antibacterial activity of guanidinylated chitosan

Ying Hu [a], Yumin Du [a,*], Jianhong Yang [a], John F. Kennedy [b,c], Xiaohui Wang [a], Liansheng Wang [a]

[a] *Department of Environmental Science, Wuhan University, Wuhan 430072, China*
[b] *Birmingham Carbohydrate and Protein Technology Group, School of Chemical sciences, The University of Birmingham, Birmingham B15 2TT, UK*
[c] *Chembiotech Laboratories, University of Birmingham Research Park, Vincent Drive, Birmingham B15 2SQ, UK*

Received 28 March 2006; accepted 10 April 2006
Available online 23 June 2006

**Abstract**

Guanidinylated chitosan derivatives with different molecular weights have been synthesized by the guanidinylation reaction of chitosan with aminoiminomethanesulfonic acid (AIMSOA); the structures of AIMSOA and guanidinylated chitosan were characterized by UV, FT-IR, fluorescence and $^{13}C$ NMR. The absorbance at 230 nm, a emission band ranging from 500 to 540 nm in emission spectrum were observed. In addition, the strong peaks at 1649, 1555, 1380 $cm^{-1}$, the distinct signals at 158.3 ppm in $^{13}C$ NMR were found. The substitution degrees of guanidinylated chitosan were confirmed by elemental analysis. In vitro antibacterial activity of guanidinium derivatives was evaluated against *Staphylococcus aureus*, *Bacillus subtilis*, *Escherichia coli* and *Pseudomonas aeruginosa*. Compared with chitosan, guanidinylated chitosan had much better antibacterial activity, whose minimum inhibitory concentrations in aqueous hydrochloric acid (pH 5.4) were 4 times lower than those of chitosan. Interestingly, guanidinylated chitosan inhibited the growth of *S. aureus* and *B. subtilis* at pH 6.6. The antibacterial activity of guanidinylated chitosan enhanced with decreasing pH.


*Keywords:* Guanidinylated chitosan; Chitosan; Synthesis; Antibacterial activity

## 1. Introduction

Chitosan, derived from chitin, is a natural nontoxic biopolymer consisting of β-(1,4)-2-acetamido-2-deoxy-D-glucose and β-(1,4)-2-amino-2-deoxy-D-glucose units. Chitosan has attracted considerable interests for its potential beneficial biological activities, such as antimicrobial (Choi et al., 2001; Jeon, Park, & Kim, 2001; No, Park, Lee, & Meyers, 2002; Yoshihiko et al., 2003), antitumor (Suzuki, Mikami, Okawa, Tokoro, & Suzuki, 1986) activity and its immune enhancing effects (Sugano, Yoshida, Hashimoto, Enomoto, & Hirano, 1992). Many attempts have been taken up to improve the antimicrobial activity of chitosan. We have previously reported the preparation and antimicrobial effect of chitosan complexes with surfactant, such as alkyl-β-D-glucopyranoside, betaine and metals (Liu, Du, Wang, Hu, & Kennedy, 2004; Liu, Du, Wang, & Sun, 2004; Wang, Du, Fan, Liu, & Hu, 2005).

Polycation biocides have been shown to possess higher activity against bacteria. Chitosan possesses primary amino groups in its structure and the number of these amino groups is related to the rate of antimicrobial activity. The introduction of asparagine to chitosan oligosaccharide significantly improved the bactericidal activity and minimum inhibitory concentration; this probably indicates that the higher the number of amino groups, the higher the antimicrobial activity (Jeon & Kim, 2001). Guanidinium salts have attracted increasing interest in recent years. Guanidines have long been the focus of considerable attention as a ubiquitous moiety incorporated into many drugs with numerous therapeutic applications and biological activi-

* Corresponding author. Tel./fax: +86 27 6877 8501.
*E-mail address:* duyumin@whu.edu.cn (Y. Du).

0144-8617/$ - see front matter © 2006 Elsevier Ltd. All rights reserved.
doi:10.1016/j.carbpol.2006.04.015

ties, such as antidiabetic and antimicrobial drugs (Greenhill & Lue, 1993). The guanidine derivatives with antimicrobial and antifungal activity have been investigated as medical and crop protection agents and antiseptics for industry products, food and other goods for daily use. Therefore, the possibility of guanidinylated chitosan with multi-amino groups, of which there is no previous report seemed attractive.

This study therefore included attempts to expand the antibacterial pH range and improve antibacterial activity of chitosan through a convenient chemical modification. A series of chitosan guanidiniums were prepared using four different molecular weights of chitosan s and the product guanidinylated chitosans were comprehensively characterized and their antibacterial activities evaluated. In order to research the relationship between molecular weight, pH and antibacterial activity of chitosan guanidinium, the effects of molecular weight and pH on the antibacterial activity of the chitosan guanidiniums were investigated.

## 2. Experimental

### 2.1. Materials

Chitosan (CS0) with molecular weight 210 kDa was supplied by Yuhan Ocean Biochemistry Co. Ltd. (Taizhou, China). The deacetylation degree was 91.6%, which was determined by a pH titration method (Tolaimate et al., 2000). Chitosans (CS1, CS2, CS3) with different molecular weights were produced by hydrolysis of CS0 by neutral protease, derived from *Bacillus subtilis* 1.398, which was supplied by Ningxia Xiasheng Industry Co. Ltd. (Ningxia, China). The molecular weights of CS0, CS1, CS2, CS3 are listed in Table 1. Aminoiminomethanesulfinic acid was supplied by Nanle Hongye Chemical Company Ltd. (Puyang, China). Beef extract and peptone were purchased from Shanghai Chemical Agent Co. (Shanghai, China). All other chemicals used were of analytical grade.

### 2.2. Preparation of guanidine derivatives

#### 2.2.1. Preparation of aminoiminomethanesulfonic acid (AIMSOA)

AIMSOA was prepared according to the synthetic procedure given in the literature (Kim, Lin, & Mosher, 1988) in which aminoiminomethanesulfinic acid (AIMSA) was conveniently converted to AIMSOA using peracetic acid (Findley, John, & Scanlan, 1945), which was then purified by recrystallization.

Table 1
Molecular weights of chitosans and guanidinylated derivatives

| Chitosan (before reaction) | | Molar ratio → | Guanidinylated chitosan (after reaction) | |
|---|---|---|---|---|
| Initial material | $M_w$ ($\times 10^{-4}$) | | Derivative product | $M_w$ ($\times 10^{-4}$) |
| CS0 | 21 | 1:1 | CSG1 | 16.4 |
| CS0 | 21 | 1:2 | CSG2 | 13.5 |
| CS0 | 21 | 1:3 | CSG3 | 12.5 |
| CS1 | 2.2 | 1:1 | CGM1 | 1.3 |
| CS2 | 5.6 | 1:1 | CGM2 | 3.1 |
| CS3 | 7.6 | 1:1 | CGM3 | 5.0 |

#### 2.2.2. Preparation of guanidinylated chitosan (CSG)

CS0 (2% w/v) in 0.2 mol/L hydrochloric acid was adjusted the pH value to 8–9 by 5% w/v aqueous sodium carbonate, the precipitate was washed with distilled water to pH 7.0–7.5. The excess water was removed, and the desired amount of AIMSOA (corresponding to a molar ratio of 1:1 compared with chitosan residue) was added slowly at 50 °C with stirring. The reaction was kept at 50 °C for 15 min and then it was cooled to room temperature. The mixture was poured into saturated aqueous sodium sulfate, and the precipitate was filtered off, washed thoroughly with water and ethanol, then dried under vacuum to constant weight to give product guanidinylated chitosan (CSG1). CGM1, CGM2, CGM3 were guanidinylated chitosans prepared similarly but starting with different molecular weights of chitosans (CS1, CS2, CS3).

Different substitution degrees guanidinium derivatives CSG2, CSG3 prepared from chitosan were synthesized using different amounts of AIMSOA (corresponding to a molar ration of 1:2, 1:3 compared with chitosan residue) using the same method as above.

### 2.3. Characterization

UV–vis absorption spectra were obtained using dilute aqueous solutions on a 1601 Shimadzu UV–vis spectrophotometer. FT-IR spectra were recorded in powder form in KBr discs in the range of 4000–400 $cm^{-1}$ on a Nicolet 670 FT-IR spectrophotometer. The degree of substitution was determined by elemental analysis. The elemental analysis (C, N, H) of samples was performed on a Flash Elemental Analyzer 1112 (ThermoQuest, Milan, Italy). Fluorescence, with an excitation wavelength of 405 nm, and the emission spectra were recorded in the range of 450–550 with a F-4500 fluorescence spectroscope (Hitachi, Tokyo, Japan), from 30 min after the addition of 0.25% w/v aqueous ninhydrin (2 ml) and 0.5 M NaOH (2 ml) to 0.005% w/v aqueous CSG1 (1 ml). $^{13}C$ NMR spectra were recorded on an INOVA-600 NMR 600 MHz spectrometer (Varian, Palo Alto, CA, USA). Samples were dissolved in $HCl/D_2O$. Molecular weights ($M_w$) of sample was measured by gel permeation chromatography (GPC) on connected columns (TSK G5000-PW or TSK G3000-PW), using an RI 150 (Thermoquest, San Jose, CA, USA) refractive index detector using 0.2 M $CH_3COOH$/0.1 M $CH_3COONa$ as eluent at a flow rate of 1.0 ml/min. The standards used to calibrate the column were pullulan (TOSOH, Tokyo, Japan). All data provided by the GPC system were collected and analyzed using the Jiangshen Workstation (DaLian Jiangshen, DaLian, China) software package.

### 2.4. Microorganisms and culture conditions

*Staphylococcus aureus* ATCC 25923, *E. coli* ATCC 25922 and *Pseudomonas aeruginosa* ATCC 27853, as used for antibacterial evaluation, were provided by the Typical Culture Collection Center in Wuhan University, China, and *B. subtilis* was provided by the Biology Engineering college of Hubei University of Technology, China. The cultures, obtained by growing the bacteria overnight at 37 °C in nutrient broth, were diluted with sterile normal saline (0.9%) solution, each of the culture suspensions containing ca. $10^6$–$10^7$ CFU/ml, which was used for the antibacterial test.

### 2.5. Evaluation of antibacterial activity in vitro

CSG1 (1% w/v) and CS0 in 1% w/v hydrochloric acid were adjusted to pH 5.4, by the addition of 2 mol/L NaOH. The chitosan derivatives with different $M_w$ were dissolved in 0.2 M sodium acetate–0.2 M acetic acid buffer (pH 6.0). The different pH values of guanidinylated chitosan (CSG3) dissolved in 1% (w/v) aqueous hydrochloric acid solution were adjusted by addition of 2 mol/L NaOH to 4.0, 4.4, 4.8, 5.2, 5.8, 6.2, 6.6, 7.0. All solutions were autoclaved at 121 °C for 15 min.

Minimum inhibitory concentration (MIC) determination: the above sample solutions were diluted 2-fold serially, and each of these solutions (1 ml) and nutrient agar (peptone 1%, beef extract 0.5%, NaCl 0.5%, agar 2%, pH 6, 9 ml) were mixed and poured into autoclaved petri-dishes, cooled, one loopful of microorganism suspension was spread on cooled nutrient agar, then incubated at 37 °C. 1% w/v aqueous hydrochloric acid (pH 5.4) was used as a control instead of sample. The MIC was defined as the lowest concentration of the tested sample at which the microorganism colonies were not visible with naked eye within 16–38 h. Relative inhibition time (RIT) was defined and measured as the difference between the time when microorganism colonies were visible in agar plates with and without test samples.

Biocidal activity determination: microorganism cell suspensions were diluted to $10^4$–$10^5$ cell/ml and these suspensions (100 μl) were added to the above, sterilised sample solutions (900 μl). After incubation at 37 °C for 60 min, aliquots (50 μl) were spread on nutrient agar plates in triplicate and then incubated at 37 °C for 1–2 days, and the colony forming units (CFU) were counted.

## 3. Results and discussion

### 3.1. Characterization of guanidinylated chitosan (CSG)

Mosher showed a general method for preparing mono-substituted guanidines over a decade ago (Kim et al., 1988). By this means, the synthesis of the guanidinylated chitosan was achieved by a convenient two step procedure starting from AIMSA as shown in Scheme 1; UV, IR, fluorescence and $^{13}C$ NMR analyses indicated the success of the guanidylation reaction.

UV–vis absorption spectra of AIMSOA, AIMSA and CSG1 dilute aqueous solution are shown in Fig. 1. In the UV spectrum of AIMSA, a broad absorption band appeared at 269 nm which was absent from the spectrum of AIMSOA, indicating that the AIMSA had been oxidized into AIMSOA. As a convenient guanidylating agent, the interest in aminoiminomethanesulfonic acid (AIMSOA) has recently increased since it converts primary amines to the corresponding guanidine derivative

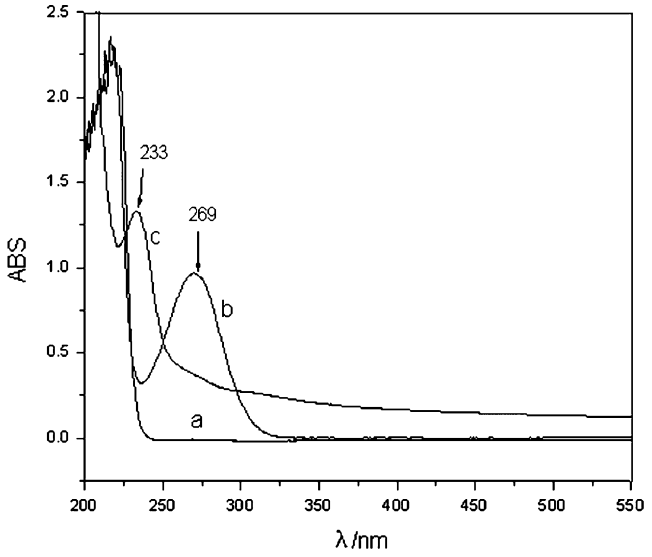
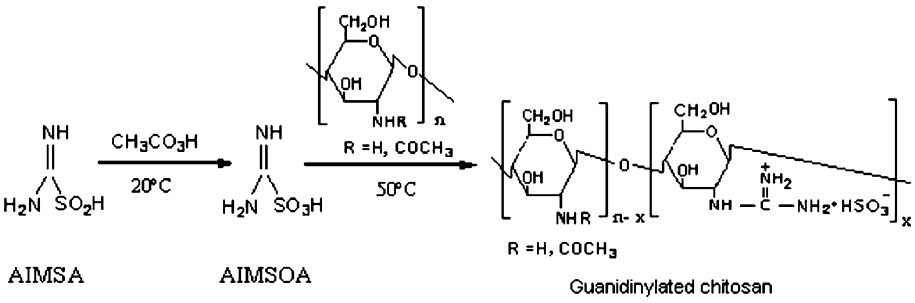



Fig. 1. UV–vis spectra of aminoiminomethanesulfonic acid (AIMSOA, a) aminoiminomethanesulfinic acid (AIMSA, b) and guanidinylated chitosan (CSG1, c).




Scheme 1. Synthesis of guanidinylated chitosan.

(Makarov et al., 1999). In the UV spectrum of CSG1, the peak at 233 nm suggested the existence of the guanidine groups (Chinese Pharmacopoeia, 1990). The key transformation in this process is displacement of the $–SO_3H$ group by an amine nucleophile.

The FT-IR spectrum of AIMSOA exhibited many differences from that of AIMSA (Fig. 2). The appearance of peaks at 1056 and 1250 $cm^{-1}$ was due to stretching vibrations of S=O; 1697 $cm^{-1}$ was due to symmetry vibrations of C=N, 3354 $cm^{-1}$ was due to stretching vibrations of N–H stretching (Cheng, 1990). All the information reflects that the AIMSA had been successfully converted into AIMSOA.

Structural changes of chitosan and its derivative were confirmed by FT-IR spectra (Fig. 3). The IR spectra of chitosan derivative showed new stronger peaks at 1649 $cm^{-1}$ and 1555 $cm^{-1}$ assigned to the stretching vibration of C=N and distortion vibration of N–H, respectively. The wide peak at 3438 $cm^{-1}$, corresponding to the stretching vibration of $–NH_2$ group and –OH group, shifted to lower frequency (3413 $cm^{-1}$). The new stronger peak at 1114 $cm^{-1}$ was assigned to the stretching vibration of C–N–C and the peak at 1320 $cm^{-1}$ was assigned to the stretching vibration of C–N, and the peak at 1597 $cm^{-1}$ that had been assigned to the bending vibration of $–NH_2$ group of chitosan had disappeared. The strong peaks at 1649, 1555, 1380 $cm^{-1}$ suggested that guanidinylation had been successful (Miller & Bischoff, 1986).

In the emission spectrum of CSG1 (Fig. 4), the weak broad emission band ranging from 500 to 540 nm was attributed to the reaction of guanidinium and ninhydrin under basic conditions (Pesez, Bartos, & Xia, 1989).

In the $^{13}C$ NMR spectra of AIMSOA, chitosan and CSG1 in $HCl/D_2O$ (Fig. 5), it can be seen that the signal of AIMSOA appeared at 168.5 ppm (Makarov et al., 1945). Comparing the $^{13}C$ NMR spectrum of chitosan with that of chitosan guanidinium, the distinct signals at 158.3 ppm were assigned to the carbons of guanidine groups (Brzozowski, Saczewski, & Gdaniec, 2002), and the $^{13}C$ NMR chemical shifts for chitosan at 56.6(C2), 60.6(C6), 70.8(C3), 75.9(C5), 78.3(C4) and 98.3(C1) (ppm) were detected. In contrast to chitosan, the signals of guanidinylated chitosan showing at 56.5(C2), 60.8(C6), 70.6(C3), 75.3(C5), 77.5(C4), 98.0(C1) (ppm) were attributed to the polysaccharide structures. The $^{13}C$ NMR spectrum again confirmed that CSG1 contained guanidine groups.

The evidences of the UV, FT-IR, fluorescence spectrum and $^{13}C$ NMR obviously support the amino groups of chitosan being partly guanidinylated.

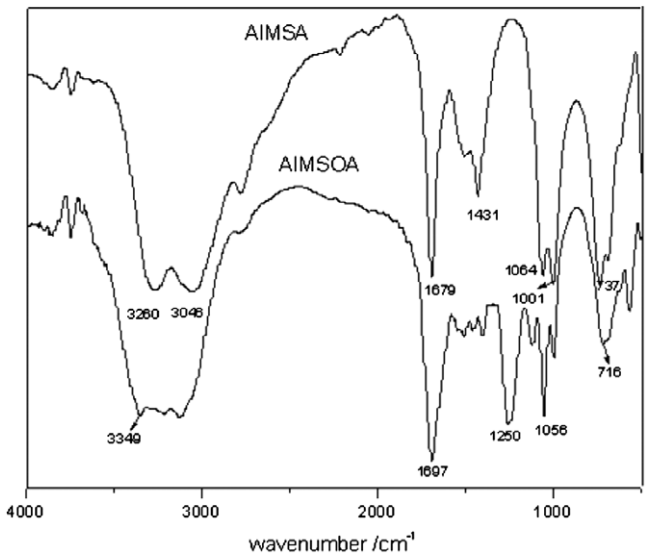



Fig. 2. FT-IR spectra of aminoiminomethanesulfonic acid (AIMSOA) and aminoiminomethanesulfinic acid (AIMSA).

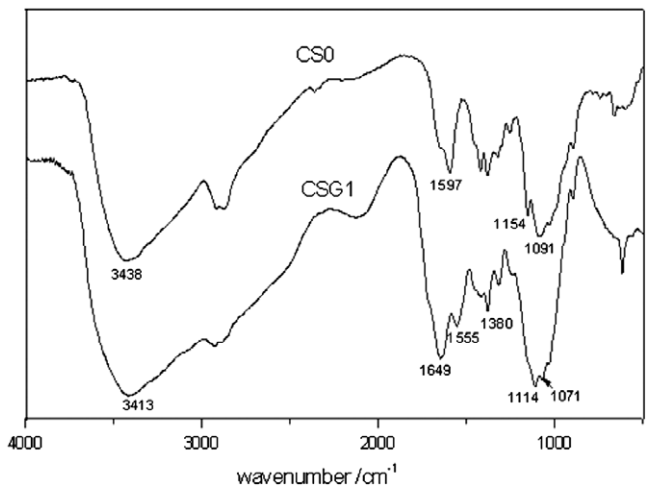



Fig. 3. FT-IR spectra of chitosan (CS0) and guanidinylated chitosan (CSG1).

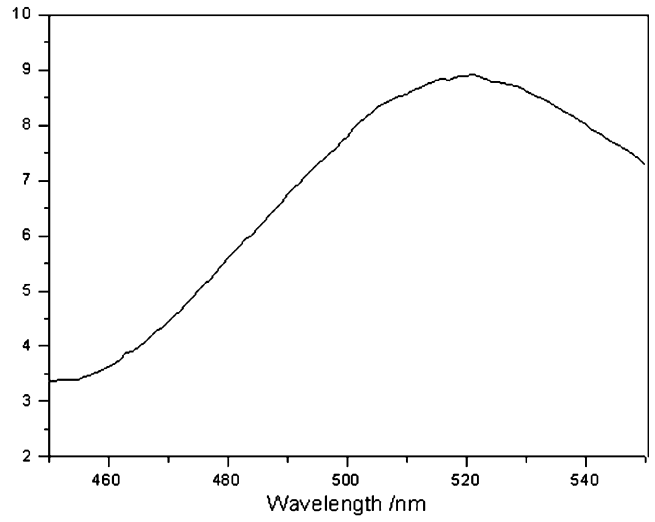



Fig. 4. Emission spectrum of 0.005% w/t guanidinylated chitosan (CSG1) aqueous solution, Ex = 405 nm, range from 450 to 550 nm.

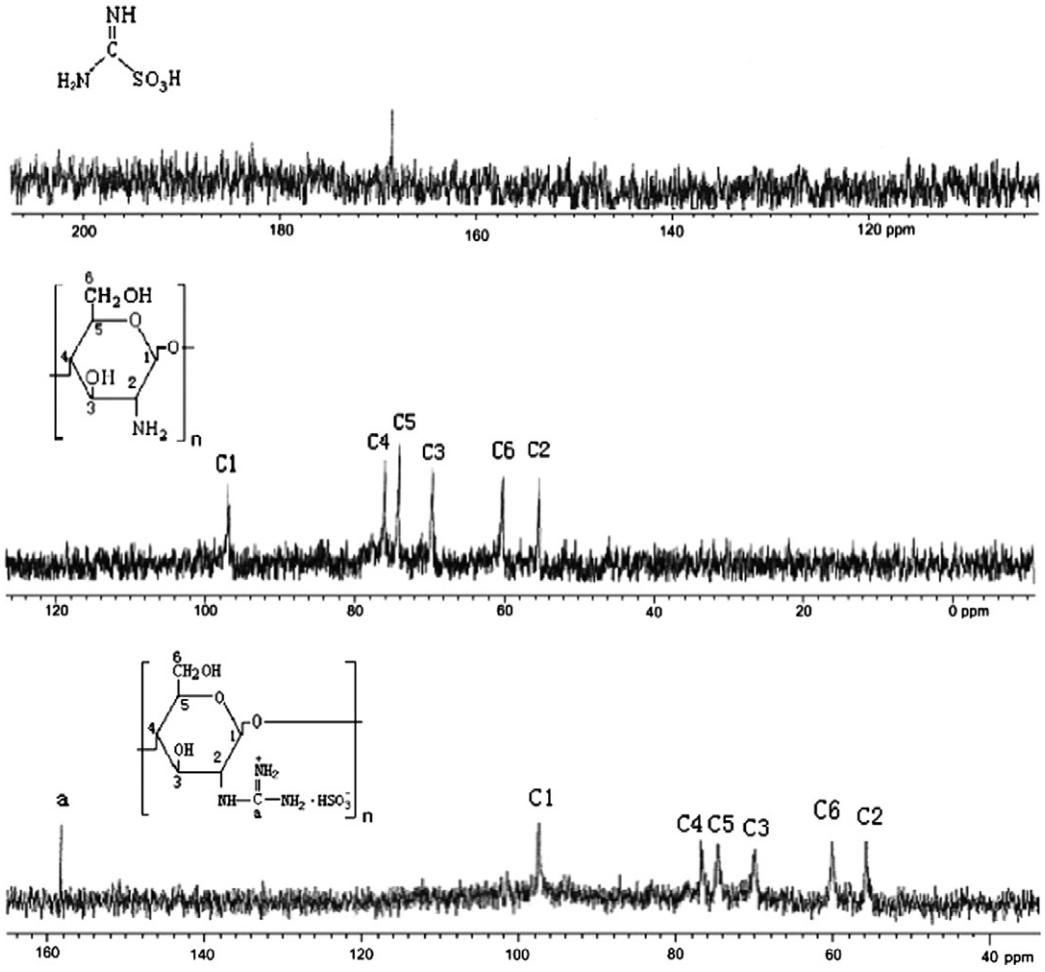



Fig. 5. $^{13}C$ NMR spectra of aminoiminomethanesulfonic acid (AIMSOA), chitosan (CS0) and guanidinylated chitosan (CSG1).

### 3.2. Molecular weight of chitosan and its derivatives and determination of substitution degree

The molecular weight of the chitosan and its derivatives plays an important role in the bioactivity such as antimicrobial activity. Table 1 shows the $M_w$ of chitosans and guanidinylated chitosan derivatives measured by GPC, and the $M_w$ of the guanidinylated chitosans decreased with increasing molar ratio of glucosamine unit to AIMSOA, this might suggest that chitosan was hydrolyzed when chitosan dissolved in AIMSOA. So, after the guanidinylation reaction, the molecular weights of the chitosan derivatives of guanidinium decreased.

The substitution degrees of the guanidinylated chitosans with different molecular weights were calculated from the elemental analysis data. As can be seen from Table 2, the C/N wt % of the guanidinylated chitosan is much lower than that of CS0. With increasing molar ratio of AIMSOA to glucosamine unit, C/N wt % in CSG slightly increased. It indicated that only a part of chitosan is converted into guanidine derivative and the substitution degrees did not increase largely because the substitution degrees of CSG1-3 were in the range of 0.25–0.30. It is probably that the decrease of pH value during the reaction process is unfavorable to the guanidinylation reaction. So, increasing the molar ratio of AIMSOA to glucosamine unit did not result in much higher substitution degree of the product derivative.

### 3.3. Antibacterial activity

The minimum inhibitory concentrations (MIC) of chitosan and its guanidinylated derivations against two gram-positive bacteria: *S. aureus* and *B. subtili* and two gram-negative bacteria: *E. coli.* and *P. aeruginosa* are shown in Table 3. In hydrochloric acid solution (pH 5.4), chitosan and guanidinylated chitosan showed effective antibacterial activity against all the microorganisms tested, and MIC values of guanidinylated chitosan were one quarter than those of chitosan. When chitosan had been converted into a guanidine derivative, the positive charge density of the derivative increased, which led to enhanced adsorption of polycation onto the negatively charged cell surface. Guanidinylated chitosan may be easier to associate with cell surface and show higher antibacterial activity. Reasonably, guanidinylated chitosan showed better antibacterial activity than chitosan. In biocidal activity data of guanidine derivatives with different $M_w$ in acetate buffer (pH

Table 2
Elemental analysis and substitution degree of chitosan CS0 and guanidinylated chitosans CGM1-3 and CSG1-3

| Sample | Molar ratio | Analysis found (Calc.) % | | | C/N | DS[a] |
|---|---|---|---|---|---|---|
| | | C | N | H | | |
| Chitosan | | | | | | |
| CS0 | | 37.0 (36.7) | 6.70 (6.96) | 7.55 (7.53) | 5.57 | |
| Guanidinylated chitosans | | | | | | |
| CGM1 | 1:1 | 31.5 (31.7) | 8.51 (8.56) | 6.49 (6.26) | 3.70 | 0.24 |
| CGM2 | 1:1 | 32.0 (32.3) | 7.71 (7.77) | 6.48 (6.37) | 4.15 | 0.15 |
| CGM3 | 1:1 | 32.0 (32.2) | 7.65 (7.64) | 6.63 (6.35) | 4.18 | 0.14 |
| CSG1 | 1:1 | 30.4 (30.6) | 8.25 (8.35) | 6.45 (6.43) | 3.68 | 0.25 |
| CSG2 | 1:2 | 31.4 (31.3) | 8.87 (9.03) | 6.81 (6.50) | 3.54 | 0.28 |
| CSG3 | 1:3 | 31.8 (31.3) | 9.20 (9.02) | 6.31 (6.33) | 3.67 | 0.30 |

[a] Substitution degree of guanidinium derivatives prepared with different mole ration of glucosamine unit to AIMSOA calculated according to the C/N wt% from the elemental analysis.

Table 3
MICs of chitosan (CS0) and guanidinylated chitosan (CSG3) in hydrochloric acid solution (pH 5.4)

| Sample (w/v, %) | $G^+$ bact. *S. aureus* | $G^+$ bact. *B. subtilis* | $G^-$ bact. *E. coli* | $G^-$ bact. *P. aeruginosa* |
|---|---|---|---|---|
| CSG3 | 0.025 | 0.025 | 0.025 | 0.025 |
| CS0 | 0.1 | 0.1 | 0.1 | 0.1 |

6.0) against *S. aureus* and *E. coli* (Table 4), all the samples showed 100% bactericidal activity against *E. coli*, CSG1 showed 100% bactericidal activity while CGM1–3 showed 99% against *S. aureus*.

Chitosan displays antibacterial activity only in an acid environment (Helander, Nurmiaho-Lassila, Ahvenainen, Rhosdes, & Roller, 2001), so the effect of pH on the antibacterial activity of chitosan derivatives was studied. In the RIT data of CSG4 with different pH against tested bacteria (Table 5), it can be found that CSG showed greater activity at lower pH. In low pH medium, chitosan and its derivative possess a lot of poly-cationic amines that interact readily with negatively charged substances at the cell surface of bacteria, such as proteins, phospholipids and fatty acids, and subsequently inhibit the growth of microorganisms. Furthermore, the guanidine derivatives had antibacterial activity under a wide range of pH values, and especially inhibited the growth of two Gram-positive bacteria *S. aureus* and *B. subtilis* at pH 6.6. However, chitosan did not show antibacterial activity at pH > 5.8.

Table 4
Biocidal activity of 0.9% w/v guanidinylated chitosans (CGM1 CGM2 CGM3 CSG1) with different molecular weights in 0.2 M acetate buffer (pH 6.0) against *S. aureus* and *E. coli*

| Sample | Viable counts (log CFU/ml) | |
|---|---|---|
| | *S. aureus* $G^+$ bact. | *E. coli* $G^-$ bact. |
| CGM1 | $2.60 \pm 0.30$ | 0 |
| CGM2 | $2.30 \pm 0.02$ | 0 |
| CGM3 | $2.30 \pm 0.01$ | 0 |
| CSG1 | 0 | 0 |
| Acetate buffer | $4.28 \pm 0.02$ | $4.34 \pm 0.01$ |

Table 5
RITs (h) of 1% w/v guanidinylated chitosan (CSG3) in hydrochloric acid solution with different pH

| Sample | pH | $G^+$ bacteria *S. aureus* | $G^+$ bacteria *B. subtilis* | $G^-$ bacteria *E. coli* | $G^-$ bacteria *P. aeruginosa* |
|---|---|---|---|---|---|
| CSG3 | 4.0 | >120 | >120 | >120 | >120 |
| | 4.4 | >120 | >120 | >120 | >120 |
| | 4.8 | >120 | >120 | >120 | >120 |
| | 5.2 | >120 | 48 | >120 | 48 |
| | 5.8 | 24 | 24 | 24 | 16 |
| | 6.2 | 24 | 24 | 24 | 16 |
| | 6.6 | 16 | 16 | 0[a] | 0[a] |
| | 7.0 | 0[a] | 0[a] | 0[a] | 0[a] |

[a] No antibacterial activity.

Till now, the mechanism of antibacterial activity of chitosan is unknown. It has been suggested that the positive charge of the amino group at the C-2 in the glucosamine monomer in chitosan allows interactions with negatively charged microbial cell membranes that lead to the leakage of intracellular constituents (Papineau, Hoover, Dnorr, & Farkas, 1991; Sudarshan, Hoover, & Knorr, 1992; Helander et al., 2001). Accordingly, adding amino groups would enhance the antibacterial activity. Nevertheless, only a few studies have been made on the antibacterial effect of increasing the number of amino groups at the C-2 position. Jeon reported that asparagine N-conjugated chitosan oligosaccharide with two positive charges strongly interacts with the carboxyl negative charges on the bacteria cell wall (Jeon et al., 2001). In the current study, an attempt was made to increase the number of amino groups by substituting amino by formamidine to obtain a guanidinylated chitosan. which showed better antibacterial activity than chitosan. Its MIC values in hydrochloric acid solution (pH 5.4) against bacteria were 4 times lower than those of chitosan. Interestingly, it inhibited the growth of *S. aureus* and *B. subtilis* at pH 6.6.

## 4. Conclusions

For the first time, guanidinylated chitosans have been successfully synthesized, and characterized by UV,

FT-IR, fluorescence spectroscopy, $^{13}C$ NMR and elemental analysis. For the first time, the antibacterial activity of guanidinylated chitosan was systematically studied. Guanidinylated chitosan showed better antibacterial activity than chitosan. The antibacterial activity of guanidinylated chitosan increased with decreasing pH. All the results imply guanidinylated chitosan will be useful as a potential new antibacterial agent.

## Acknowledgement

This work was supported by the National Natural Science Foundation of China (Grant No. 29977014).

## References

Brzozowski, Z., Saczewski, F., & Gdaniec, M. (2002). Synthesis, molecular structure and anticancer activity of 1-allyl-3-amino-2-(4-chloro-2-mercaptobenzenesulphonyl) guanidine derivatives. *European Journal of Medicinal Chemistry, 37*, 285–293.

Cheng, Z. S. (1990). *Jingxi Huaxuepin Huaxue*. Huadong Huagong Xueyuan Press, p. 477.

Chinese Pharmacopoeia 1990, Pharmacopeia Commission of PRC, Beijing: Xueyuan Huagong Press, People's Medical Publishing House, 1990, p.498.

Choi, B. K., Kim, K. Y., Yoo, Y. J., Oh, S. J., Chio, J. H., & Kim, C. y. (2001). In vitro antimicrobial activity of a chitooligosaccharide mixture against *Actinobacillus actinomycetimcomitans* and *Streptococcus* mutans. *International Journal of Antimicrobial Agents, 18*, 553–557.

Findley, T. W., John, D. S., & Scanlan, T. (1945). Epoxidation of unsaturated fatty materials with peracetic acid in glacial acetic acid solution. *Journal of American Chemistry, 67*, 412–414.

Greenhill, J. L., Lue, P. (1993). In G. P., Ellis, D. K., Luscombe (Eds.), Progress in medicinal chemistry. Vol. 30, Elsevier Science: New York, chapter 5.

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

Jeon, Y. J., & Kim, S. K. (2001). Effect of antimicrobial activity by chitosan oligosaccharide N-conjugated with asparagine. *Journal of Microbiology and Biotechnology, 11*(2), 281–286.

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

Kim, K., Lin, Y. T., & Mosher, H. S. (1988). Monosubstituted guanidines from primary amines and aminoiminomethanesulfonic acid. *Tetrahedron Letters, 29*, 3183–3186.

Liu, H., Du, Y. M., Wang, X. H., Hu, Y., & Kennedy, J. F. (2004). Structure characterization and antimicrobial activity of chitosan/betaine derivative complex. *Carbohydrate Polymers, 56*, 291–297.

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

Makarov, S. V., Mundoma, C., Penn, J. H., Petersen, J. L., Svarovsky, S. A., & Simoyi, R. H. (1999). *Ingorganica Chemica Acta, 286*, 149–154.

Miller, A. E., & Bischoff, J. J. (1986). A facile conversion of amino acids to guanidino acids. *Synthesis, 9*, 777–779.

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

Papineau, A. M., Hoover, D. G., Dnorr, D., & Farkas, D. F. (1991). Antimicrobial effect of water-soluble chitosan s with high hydrostatic pressure. *Food Biotechnology, 5*, 45–57.

Pesez, M., Bartos, J., Eds; Xia, J.R. Interprete, Youji Huahewu Ji Yaowu De Bisefa He Yingguang Fenxifa. Beijing: Zhongguo Renmin Gongan Daxue Press, 1989, p. 209.

Sugano, M., Yoshida, K., Hashimoto, M., Enomoto, K., & Hirano, S. (1992). Hypocholesterolemic activity of partially hydrolyzed chitosan s in rats. In C. J. Brine, P. A. Sandford, & J. P. Zikakis (Eds.), *Advances in chitin and chitosan* (pp. 472–478). London: Elsevier.

Sudarshan, N. R., Hoover, D. G., & Knorr, D. (1992). Antibacterial action of chitosan. *Food Biotechnology, 6*, 257–272.

Suzuki, K., Mikami, T., Okawa, Y., Tokoro, A., & Suzuki, M. (1986). Antitumor effect of hexa-N-acetychtohexaose and chitohexaose. *Carbohydrate Research, 151*, 403–408.

Tolaimate, A., Desbrieres, J., Rhazi, M., Alagui, A., Vincendon, M., & Vottero, P. (2000). On the influence of deacetylation process on the physicochemical characteristics of chitosan from squid chitin. *Polymer, 41*, 2463–2469.

Wang, X. H., Du, Y. M., Fan, L. H., Liu, H., & Hu, Y. (2005). Chitosan-metals complexes as antimicrobial agent: synthesis, characterization and structure-activity study. *Polymer Bulletin, 55*, 105–113.

Yoshihiko, O., Mayumi, S., Takahito, A., Hiroyuki, S., Yoshihiro, S., Ichiro, N., et al. (2003). Antimicrobial activity of chitosan with different degrees of acetylation and molecular weights. *Biocontrol Science, 8*, 25–30.