

## ALTERED ENZYMATIC ACTIVITY OF LYSOZYMES BOUND TO VARIOUSLY SULFATED CHITOSANS\*

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**Abstract** The purpose of this research is to investigate the effects of the variously sulfated chitosans on lysozyme activity and structure. It was shown that the specific enzymatic activity of lysozyme remained almost similar to the native protein after being bound to 6-O-sulfated chitosan (6S-chitosan) and 3,6-O-sulfated chitosan (3,6S-chitosan), but decreased greatly after being bound to 2-N-6-O-sulfated chitosan (2,6S-chitosan). Meanwhile, among these sulfated chitosans, 2,6S-chitosan induced the greatest conformational change in lysozyme as indicated by the fluorescence spectra. These findings demonstrated that when sulfated chitosans of different structures bind to lysozyme, lysozyme undergoes conformational change of different magnitudes, which results in corresponding levels of lysozyme activity. Further study on the interaction of sulfated chitosans with lysozyme by surface plasmon resonance (SPR) suggested that their affinities might be determined by their molecular structures.

**Keywords:** Lysozyme; Sulfated chitosan; Activity; Structure; Regioselective modification.

## INTRODUCTION

Chitosan is one of the most important natural polymers in terms of its wide applications in the medical fields, such as immunostimulation, wound healing, tissue engineering and drug delivery<sup>[1–5]</sup>. Over the last decades, the researchers have discovered that the chemical modifications of chitosan could not only improve its physicochemical properties, but also give it more functions in biomedical applications<sup>[2, 6]</sup>.

More and more attentions have been paid to the sulfation of chitosan and its potential in biomedical applications. Sulfated chitosans, with a similar molecular structure to heparin, can achieve high anticoagulant and anti-HIV activities, as well as bind to lysozyme specifically<sup>[7–11]</sup>. Thus, it is generally regarded that sulfated chitosans act in the same way as heparin, which has the specific affinity to proteins such as antithrombin III, gp120 and lysozyme, and is consequently capable of regulating those proteins' activities<sup>[6, 7, 12–14]</sup>. It was found that the molecular structure of sulfated chitosans, especially the positions of sulfate groups on chitosan, could play an important role in influencing the anticoagulant and anti-HIV activities<sup>[9, 14]</sup>. However, till now, relatively few works have explained the effects of sulfated chitosans on protein structure and activity.

Lysozyme is a natural enzyme and can bind with chitosan and sulfated chitosans specifically<sup>[11, 15–17]</sup>. It

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serves as an innate immune response antibiotic and an important defence molecule mainly by hydrolyzing the bacterial cell wall<sup>[18–20]</sup>. Recent investigations show that the application of lysozyme is also effective in the treatment of HIV infection and cancer<sup>[21–24]</sup>. Lysozyme is a small protein with simple structure, and its bioactivity can be easily detected. Therefore, lysozyme has been a model protein in the study on protein structure and the regulation of protein activity. Here, we measured the hydrolytic activity and the fluorescence of lysozyme after binding to regioselectively sulfated chitosans to study the effects of variously sulfated chitosans on lysozyme activity and structure. Moreover, to evaluate the influence of different sulfated chitosans on lysozyme function, the affinity of lysozyme to sulfated chitosans was also studied.

## EXPERIMENTAL

### Materials

Chitosan oligosaccharide with average molecular weight less than 10 kDa and degree of deacetylation (DD) 0.87 was from Jinan Haidebei Marine Bioengineering Co. All other chemical and biological reagents were from Sinopharm Chemical Reagent Co. or Fluka. Chemicals were not purified, dried or pretreated unless specified. *N,N*-dimethylformamide (DMF) was dried over anhydrous  $\text{MgSO}_4$  and subsequently distilled under vacuum.

### Synthesis and Characterization of 6-*O*-sulfated Chitosan (6S-chitosan), 2-*N*-6-*O*-sulfated Chitosan (2,6S-chitosan) and 3,6-*O*-sulfated Chitosan (3,6S-chitosan)

The regioselective modification of sulfate groups on chitosans was performed as reported previously<sup>[11]</sup>. Figure 1 shows the structures of the products and the reaction conditions. The products were dialyzed and lyophilized for the following experiments.  $^{13}\text{C}$ -NMR spectra were recorded at 125 MHz on a Bruker AVANCE DRX-500 spectrometer at 300 K in  $\text{D}_2\text{O}$  with tetramethylsilane as internal reference. IR spectra were recorded on a Nicolet Nexus Fourier Transform Infrared Spectrometer. Elemental analysis was performed on a Vario ELIII CHNSO elemental analyzer (Elementar). The data from these experiments showed that the structures and degrees of sulfation of the sulfated chitosans were as reported previously<sup>[11]</sup>.

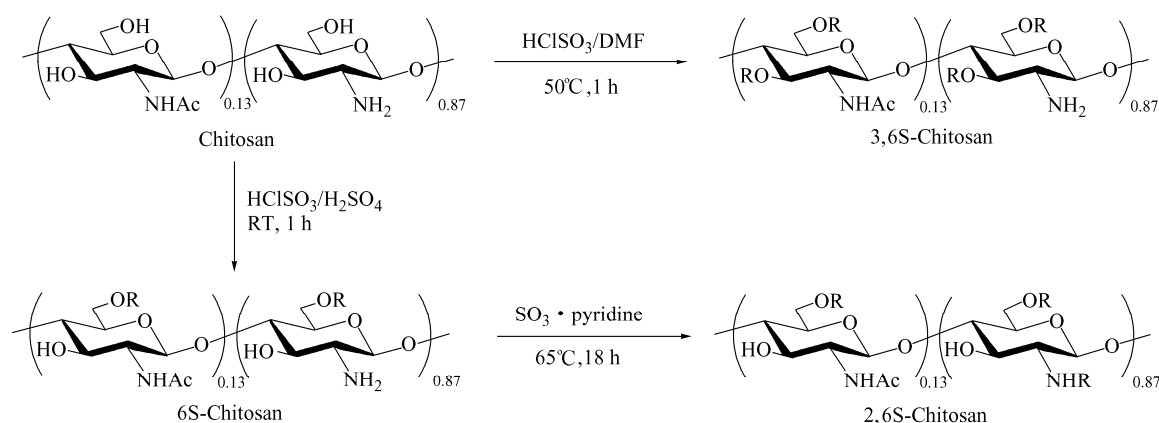


**Fig. 1** Preparation of regioselective sulfated chitosans and the chemical structures of chitosan, 6S-chitosan, 2,6S-chitosan and 3,6S-chitosan ( $\text{R} = \text{SO}_3\text{Na}$  or  $\text{H}$ )

### Assay of Lysozyme Activity

Lysozyme and sulfated chitosans were mixed and centrifuged according to Yuan *et al.*<sup>[11]</sup>. The centrifuged precipitates were resuspended in the reaction buffer (0.1 mol/L sodium phosphate, 0.1 mol/L  $\text{NaCl}$ , pH 7.5, containing 2 mmol/L sodium azide) with fluorescent substrate (EnzChek<sup>®</sup> Lysozyme Assay Kit, Invitrogen), and incubated at  $37^\circ\text{C}$  for 1 h. The reaction products of the fluorescent substrate were measured at Ex485/Em518, and the activity of the lysozyme was calculated from the standard curve. Total activity was the activity of lysozyme in the centrifuged precipitates. Specific activity was calculated by dividing lysozyme activity by the amount of proteins in the precipitates.

### **Fluorescence Determination of Lysozyme**

The complexes of lysozyme-sulfated chitosans were centrifuged and resuspended in the reaction buffer for the lysozyme activity assay without the addition of fluorescent substrate. The lysozyme concentration in the suspensions for all the samples was adjusted to  $0.4 \text{ g L}^{-1}$  prior to fluorescence determination. Fluorescence spectra of the different samples by excitation at 280 nm were detected in the range 290–450 nm by CRT-950 (Sanke Co). The excitation and emission slits were set at 5 nm and 2 nm, respectively.

### **Measurement of Lysozyme Binding to Sulfated Chitosans by Surface Plasmon Resonance (SPR)**

Lysozyme immobilization on gold was done by the method of Lee *et al.*<sup>[25]</sup>. Immobilization of lysozyme ( $1 \text{ g L}^{-1}$ ) on the surface was measured by SPR (SR7000DC, Reichert, USA). Lysozyme was bound to the disuccinimidyl carbonate (DSC)-activated poly(oligo(ethylene glycol) methacrylate) (poly(OEGMA)) in amounts greater than  $500 \text{ ng cm}^{-2}$ , while only *ca.*  $2 \text{ ng cm}^{-2}$  was bound to a surface where the poly(OEGMA) was not activated.

SPR studies of the interactions between lysozyme and the sulfated chitosans were carried out by the injection of the different sulfated chitosan solutions under constant flow ( $50 \mu\text{L min}^{-1}$ ), followed by washing with Tris-HCl ( $50 \text{ mmol/L}$ , pH 7.4) for 2 min. The relative amounts of sulfated chitosans adsorbed on the gold surface were estimated from the shift of the resonance angle.

### **Measurement of Lysozyme Binding to Sulfated Chitosans**

In order to achieve more sensitive signal in measuring protein amount, the lysozyme was labeled by fluorescent agent. Fluorescein isothiocyanate (FITC) labeled lysozyme was prepared according to Chen *et al.* with some modifications<sup>[26]</sup>. Briefly, FITC was dissolved in anhydrous DMF at a concentration of  $10 \text{ g L}^{-1}$ .  $100 \mu\text{L}$  FITC solution was mixed with lysozyme ( $10 \text{ g L}^{-1}$ , pH 8.5) and stirred at room temperature for 60 min.  $0.1 \text{ mL}$  hydroxylamine-HCl was added to the solution and stirred for 10 min at room temperature to stop the reaction. Free FITC and protein were then separated on a Sephadex G-50 column pre-equilibrated with  $0.1 \text{ mol/L}$  sodium bicarbonate (pH 8.5). The proteins were concentrated and dissolved in Tris-HCl buffer ( $10 \text{ mmol/L}$ , pH 7.1) after ultrafiltration (Amicon Ultra, Millipore, USA).

FITC-lysozyme and sulfated chitosans were mixed in  $100 \mu\text{L}$  Tris-HCl buffer ( $10 \text{ mmol/L}$ , pH 7.1) with the final concentrations at  $0.4$  and  $1.6 \text{ g L}^{-1}$ , respectively. After incubation at room temperature for 5 min, the suspensions were centrifuged at  $12000 \times g$  for 10 min. The solids were then resuspended, and fluorescence was measured at Ex485/Em518. The quantities of lysozyme bound to the sulfated chitosans were calculated from a standard curve.

## **RESULTS AND DISCUSSION**

### **The Effect of Sulfated Chitosan on the Enzymatic Activity of Lysozyme**

EnzChek Lysozyme Assay kit was used to measure the activity of the sulfated chitosan bound lysozyme in this study since it provides a sensitive and stable assay of lysozyme activity<sup>[27, 28]</sup>. In this research, total activity and specific activity were both measured to understand the effect of the sulfated chitosan on lysozyme hydrolysis activity. Total activity was the activity of all lysozyme molecules bound to the sulfated chitosans. Therefore, total activity was decided not only by the binding ability of lysozyme with the sulfated chitosans, but also by the hydrolysis activity of each lysozyme molecule bound to the sulfated chitosans. While specific activity only reflected the hydrolysis activity of each lysozyme molecule bound to the sulfated chitosans. After incubating with the fluorescent substrate at  $37^\circ\text{C}$ , all the chitosan-bound lysozyme samples appeared to have hydrolytic activities. However, the activities varied, with 6S-chitosan showing the highest total hydrolysis of the substrate (a total activity calculated at  $336.8 \pm 12.3 \text{ U}$ ) and 2,6S-chitosan the lowest ( $29.8 \pm 1.6 \text{ U}$ ) (Fig. 2). The total activities of lysozyme bound to 2,6S-chitosan and 3,6S-chitosan were only *ca.* 9%–14% of that to 6S-chitosan.

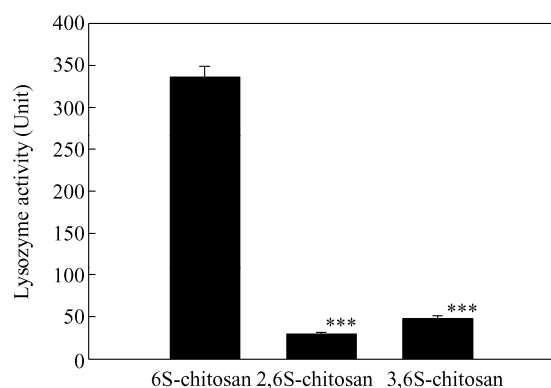


**Fig. 2** Total activity of lysozyme bound to the sulfated chitosans

The original activity of lysozyme before binding with the sulfated chitosans was 512 units. Unbound lysozymes were removed by centrifugation. Each column represents the mean of separate experiments with bar as SE ( $n = 3$ ). Statistical significances were assessed relative to 6S-chitosan, \*\*\* for  $P < 0.001$ .

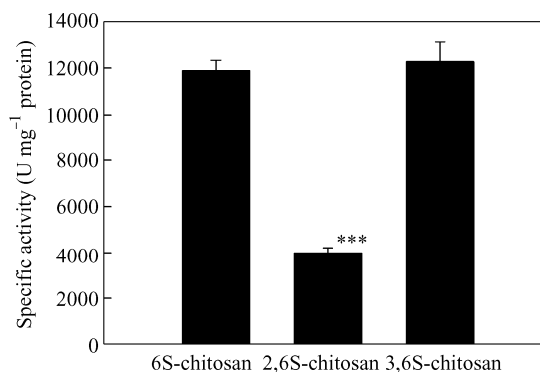


**Fig. 3** Specific activity of lysozyme bound to the sulfated chitosans

The specific activity of native lysozyme was  $(12803 \pm 239) \text{ U mg}^{-1}$ . Each column represents the mean of separate experiments with bar as SE ( $n = 3$ ). Statistical significances were assessed relative to 6S-chitosan, \*\*\* for  $P < 0.001$ .

The specific activity of an enzyme displays the rate of the reaction that catalyzed by the enzyme. It can represent the activity of an individual enzyme molecule. The activity of lysozyme bound to sulfated chitosans was further described by their specific activity in our study. Although the total activity of the bound lysozyme to 6S-chitosan was much higher than that to the other two sulfated chitosans, the specific activity of 6S-chitosan-bound lysozyme  $((11899 \pm 435) \text{ U mg}^{-1} \text{ protein})$  was not significantly different from that of 3,6S-chitosan-bound lysozyme  $((12282 \pm 846) \text{ U mg}^{-1} \text{ protein})$ ; both specific activities were similar to that of the native lysozyme  $((12803 \pm 239) \text{ U mg}^{-1} \text{ protein})$ . However, the specific activity of lysozyme bound on 2,6S-chitosan was only  $(3967 \pm 213) \text{ U mg}^{-1} \text{ protein}$  (Fig. 3), *i.e.* about 31% of native lysozyme. These results suggest that sulfate groups on positions 3 and 6 of chitosan has little effect on altering the specific activity of bound lysozyme, while sulfate groups on position 2 affect the specific activity of lysozyme molecule adversely.

#### The Effect of Sulfated Chitosan on the Structure of Bound Lysozyme

The alteration of protein activity is generally caused by the structural change. Change of protein structure can be investigated by fluorescence due to the amino acid residues. Tryptophans in lysozyme have strong fluorescence emission at about 340 nm when excited at 280 nm. The intensity and shift of the fluorescence emission can reflect changes of lysozyme structure; this has been used in studies of interactions with various molecules<sup>[29–31]</sup>. It was observed that there was almost no fluorescence emission from the sulfated chitosans when excited at 280 nm, whereas fluorescence of high intensity (about 630.0) was seen from native lysozyme (Fig. 4). After binding with the sulfated chitosans, the fluorescence emitted by lysozyme decreased to different extents. For 6S-chitosan-lysozyme, the fluorescence (about 616.3) was little different from that of native lysozyme, and 3,6S-chitosan inhibited the fluorescence only slightly (about 564.7). However, 2,6S-chitosan showed a strong inhibition. The fluorescence intensity of lysozyme bound to 2,6S-chitosan was 464.2, about 70% of the native protein, and the fluorescence peak of lysozyme had a 3 nm shift. These results suggest that 6S-chitosan has little effect on lysozyme structure, and 3,6S-chitosan may change the lysozyme structure slightly. As expected, 2,6S-chitosan appears to change the structure significantly. Such conformational change of lysozyme explains the difference in lysozyme activity after bound to variously sulfated chitosans. The change of protein structure can also affect the affinity of lysozyme to the sulfated chitosans.

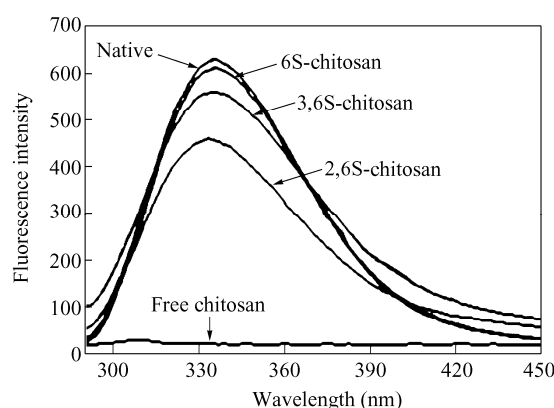


**Fig. 4** Effect of the sulfated chitosans on the fluorescence of lysozyme  
The arrow shows the sequence of the sulfated chitosans with the fluorescence intensity of the peaks from high to low. Free chitosan means chitosan not bound to lysozyme, which served as control.

#### Lysozyme Affinity to Various Sulfated Chitosans

The interaction of proteins with carbohydrate can be well studied by SPR<sup>[32]</sup>. In this research, different affinities of these sulfated chitosans to lysozyme were observed by SPR measurements (Table 1). A  $K_a$  value (association rate constant) for 6S-chitosan of  $6.32 \times 10^3 \text{ (mol/L)}^{-1} \text{ s}^{-1}$  was observed, which was about 2–3 fold greater than for 2,6S-chitosan and 3,6S-chitosan. While  $K_d$  value (dissociation rate constant) for 6S-chitosan was only about 50% that of the other two. Therefore, the  $K_D$  value (equilibrium dissociation constant, the ratio of kinetic rate constants,  $K_d/K_a$ ) of 6S-chitosan ( $0.86 \times 10^{-7} \text{ mol/L}$ ) was 4–6 times less than that of 2,6S-chitosan and 3,6S-chitosan ( $3.57 \times 10^{-7} \text{ mol/L}$  and  $5.39 \times 10^{-7} \text{ mol/L}$ , respectively), which indicated that 2,6S-chitosan and 3,6S-chitosan with a sulfate group elsewhere than on position 6 have strongly decreased affinity to lysozyme. 2,6S-chitosan might reduce the affinity to lysozyme by altering the protein structure, while the sulfate group on position 3 of 3,6S-chitosan might disturb its binding with lysozyme. The different affinities of variously sulfated chitosans then might result in the significant difference in the amount of lysozyme bound to these sulfated chitosans. Results showed that 6S-chitosan bound  $(28.3 \pm 1.3) \mu\text{g}$  lysozyme; 2,6S-chitosan and 3,6S-chitosan bound only  $(7.5 \pm 0.1) \mu\text{g}$  and  $(3.9 \pm 0.6) \mu\text{g}$ , respectively, *i.e.* about 26.5% and 13.8% of the amount bound to 6S-chitosan (Fig. 5).

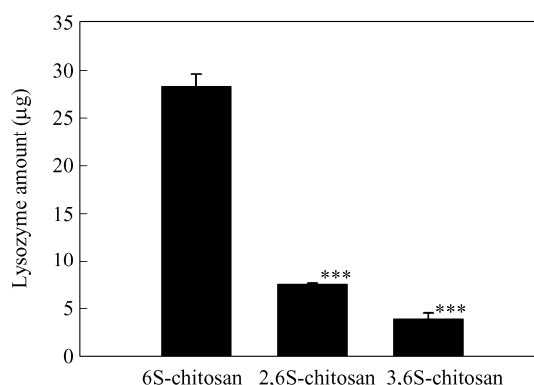


**Fig. 5** Amount of lysozyme binding to the sulfated chitosans  
Each column represents the mean of separate experiments with bar as SE ( $n = 3$ ). Statistical significances were assessed relative to 6S-chitosan, \*\*\* for  $P < 0.001$ .

**Table 1.** The interaction between lysozyme and sulfated chitosans by SPR measurement

| Sulfated chitosan | $K_a \times 10^{-3} ((\text{mol/L})^{-1} \text{s}^{-1})$ | $K_d \times 10^3 (\text{s}^{-1})$ | $K_D \times 10^7 (\text{mol/L})$ |
|-------------------|----------------------------------------------------------|-----------------------------------|----------------------------------|
| 6S-chitosan       | 6.32                                                     | 0.54                              | 0.86                             |
| 2,6S-chitosan     | 3.44                                                     | 1.23                              | 3.57                             |
| 3,6S-chitosan     | 2.04                                                     | 1.10                              | 5.39                             |

The above results suggest that 6S-chitosan, which has only hydroxyl group sulfated at position 6, promotes lysozyme binding strongly and has no apparent negative effect on lysozyme activity and structure; 2,6S-chitosan and 3,6S-chitosan, which have additional sulfate groups on position 2 or 3 besides position 6, both inhibit lysozyme adsorption. 3,6S-chitosan inhibits lysozyme binding strongly, while 2,6S-chitosan decreases lysozyme activity greatly. Generally, specific binding of proteins to polysaccharides is due to the recognition of specific structural features. The number of such features and their interaction energies determine the strength of binding. Thus there are two possible reasons for the differences in lysozyme binding among 2,6S-chitosan, 3,6S-chitosan and 6S-chitosan. One is that the sulfation of position 6 results in an optimal structure/conformation of chitosan for binding to lysozyme. Therefore, 6S-chitosan binds high amount of lysozyme. 2,6S-chitosan and/or 3,6S-chitosan may have different conformations from 6S-chitosan, and are thus unable to bind efficiently to lysozyme. The other is that 2,6S-chitosan can even change the conformation of lysozyme, thereby decreasing the binding energy and reducing lysozyme adsorption.

As to the effect on hydrolytic activity of lysozyme, 2,6S-chitosan, 3,6S-chitosan and 6S-chitosan also have different behaviors. No matter how strong or weak adsorption of lysozyme appears in 3,6S-chitosan and 6S-chitosan, lysozyme bound on either of them displays similar specific activity. However, if sulfation is on both position 2 and position 6, the resulting chitosan changes the lysozyme structure and decreases the lysozyme activity. It might be due to the sulfation on position 2 of chitosan. Originally, there is an amino group on position 2 of chitosan, which can carry positive charge in physiological environment. After sulfation, an additional sulfate group is linked on nitrogen at position 2 and turns the charge into a negative one. Then, such change strongly affects the interaction of 2,6S-chitosan and lysozyme, and finally leads to a significant change of lysozyme structure and a great decrease of lysozyme activity.

Since the variously sulfated chitosans have different effects on lysozyme activity, structure and adsorption, it is important to evaluate the application of sulfated chitosans in biomedical fields. As a multi-functional protein and an important defence molecule, lysozyme should maintain its activity when treated by sulfated chitosans. Therefore, although great potentials of sulfated chitosans have been revealed in the applications of anticoagulation and anti-HIV, more detailed studies of the effect of sulfated chitosans on protein structure and activity, including the structure and concentration of the sulfated chitosans, should be carried out *in vivo* in the future.

## CONCLUSIONS

Variouly sulfated chitosans with regioselective modification of sulfated groups on positions 2, 3 and 6, showed different effects on lysozyme activity, structure and adsorption. Among them, lysozyme bound on 6S-chitosan and 3,6S-chitosan had similar specific activity with the native protein, while 2,6S-chitosan greatly decreased lysozyme activity. The results of the fluorescence emission of lysozyme bound on the sulfated chitosans suggested that the effect of sulfated chitosans on lysozyme specific activity might be due to the structural change caused by these sulfated chitosans. Meanwhile, 6S-chitosan, 2,6S-chitosan and 3,6S-chitosan also exhibited different affinities to lysozyme. Among the three, 6S-chitosan showed the highest affinity to lysozyme. Lysozyme binding on 2,6S-chitosan and 3,6S-chitosan was much less than that on 6S-chitosan. These effects can be attributed to structural differences among these sulfated chitosans and the conformational change in lysozyme after binding to the sulfated chitosans. The interactions of lysozyme with the sulfated chitosans are presumably related to the positioning of the sulfate groups. The sulfate group at position 6 appears to be mainly responsible for binding to lysozyme, while additional sulfate groups at positions 2 and 3 reduced the binding on 6S-chitosan.

Moreover, the additional sulfate group at position 2 may also decrease the specific activity of lysozyme. This study will benefit the researches in the application of sulfated chitosans in biomedical fields.

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