INTRODUCTION

Biopolymers, chitosan is the deacetylated form of chitin and composed of glucosamine, known as (1–4)-2-amino-2-deoxy-d-glucose. Chitosan has three types of reactive functional groups, an amino group as well as both primary and secondary hydroxyl groups at the C-2, C-3 and C-6 positions, respectively (10). This special structure makes it exhibit chelation with various metal ions (4). Muzzarelli (21) pointed out that chitosan combines with metal ions by three forms: ion exchange, sorption and chelation. Chitosan has been broadly used for the sorption of heavy metal ions (7, 14, 25). Further physical and chemical modifications of chitosan have been made to improve the selectivities and the capacities for metals ions (6, 11, 16, 22). Chitosan is also characterized by weak diffusion properties: long contact times are required to reach equilibrium. Sorption capacity can be controlled by sorbent particle size (11). Due to the low porosity of chitosan, sorption performances are frequently controlled by mass transfer resistance. To reduce this resistance to mass transfer, chitosan gel beads have been developed to expand the polymer structure and reduce its crystallinity (1, 12, 13). However, these treatments results in either a decrease of the number of available sorption sites (cross-linking treatment), or the volumetric sorption capacity (the percentage of water in the gel beads can reach 95 %) (24). Controlled drying can increase the volumetric sorption capacity (23). Another possibility for increasing this volumetric sorption capacity is the grafting of supplementary functional groups (5).

MATERIALS AND METHODS

Neutral proteinase (20,000 U/g) was a commercial enzyme, of food-grade, obtained from Wuxi enzyme reagent Factory (Jiangsu, China). Chitosan was provided by Yuhuan Ocean Biochemical Co. Lit (Zhejiang, China), the molecule weight of which was 91,000. TPP abbreviated from Sodium polyphosphate was purchased from Dongsheng chemical reagent Factory (zhejiang, China). All other chemicals were of analytical grade and no further purification was required.

PREPARATION OF CHITOSAN NANO-PARTICLES

20 mg chitosan was dissolved in 40 ml of 2.0 % (v/v) acetic acid. 20 ml of 0.75 mg/ml TPP was dropped into the beaker. Then the solution was treated with super-filtration membrane to remove the residual TPP. Chitosan nano-particles were stored up in the distilled water.
Morphology and structure characterization of chitosan nano-particles

Chitosan nano-particles were gold coated using a Hitachi coating unit IB-2 coater under a high vacuum, 0.1 Torr, high voltage, 1.2 kV and 50 mA. Coated samples were examined using a XL30-SEM scanning electron microscope to characterize the morphology and size of nano-particles.

FT-IR of chitosan nano-particles were taken with KBr pellets on Nicolet Nexus 670 Spectrum FT-IR. The samples were chitosan, chitosan nano-particles, chitosan nano-particles adsorbed neutral proteinase respectively.

Sorption kinetics experiments

Batch experiments for determination of kinetics of neutral proteinase on chitosan nano-particles were carried out using a continuously stirred glass vessel. Different concentrations of different mean particle sizes chitosan nano-particles solutions were brought in contact with 1.0 ml neutral proteinase solutions under continuous stirring, at temperature (30~60°C). The pH was 7.0. The initial concentration of neutral proteinase was varied to investigate their effect on the sorption kinetics. During the kinetic experiments, samples were withdrawn at fixed time intervals, filtered, and analyzed with an UV/visible spectrometer 751 (Shanghai, China).

Equilibrium experiments

Batch equilibrium experiments were carried out using chitosan nano-particles as sorbent. A series of flasks containing neutral proteinase solutions of varying concentrations prepared from neutral proteinase and a fixed concentration of chitosan nano-particles were agitated in a rotary shaker at room temperature. Neutral proteinase uptake experiments were conducted under pH 7.0. After equilibration, neutral proteinase solutions were filtered and analyzed. Neutral proteinase-free and sorbent-free blanks were used as controls. Amounts of neutral proteinase taken up by the sorbent in each flask were determined by the following mass balance equation:

\[
Q = \frac{V(C_0 - C_e)}{W}
\]

where \(Q\) is the sorption capacity (mg/g), \(C_0\) and \(C_e\) are, respectively, the initial and solution phase neutral proteinase concentration at equilibrium (mg/l), \(V\) the solution volume (l), and \(W\) the mass of sorbent (g).

Results and Discussion

Characterization of chitosan nano-particles

Size and morphology of chitosan nano-particles

The preparation of chitosan nano-particles was based on an ionic gelation interaction between positively-charged chitosan and negatively-charged tri-polyphosphate (2, 15). Chitosan nano-particles prepared in the experiment exhibit white powder shape, and were insoluble in water, dilute acid, and alkalescent solution. Results were followed in Fig. 1.

Surface functional groups of chitosan nano-particles

Tri-polyphosphate has also been selected as a possible cross-linking agent for the preparation of chitosan gel beads by the coagulation/neutralization effect. Spherical chitosan tri-polyphosphate chelating beads had been prepared and applied in the field of metal ions adsorption due to the enhanced intra-particular diffusion and excellent uptake capacity (18). In this work, in order to increase the sorption capacity of chitosan, chitosan nano-particles were prepared by ionic gelation of chitosan and tri-polyphosphate.

FT-IR spectra of chitosan and chitosan nano-particles have been analyzed, and results showed that the CONH\(_2\) and NH\(_2\) groups of chitosan are both slightly cross-linked with a sodium polyphosphate molecule (Fig. 2 and 3) (19). Neutral proteinase-adsorbed nano-particles were formed by sorption of neutral proteinase. As could be seen from the IR spectrum of chitosan nano-particles, the peak indicating P=O stretching at 1217 cm\(^{-1}\) appeared (18), but disappeared for the nano-particles after adsorbing neutral proteinase due to the hydrogen bond between neutral proteinase and phosphoric groups. The peaks at 1066 cm\(^{-1}\),
cm⁻¹ (OH) in the spectrum of neutral proteinase adsorbed nanoparticles turned sharper in Fig. 4. This behavior reflected the interaction between the amino groups and neutral proteinase. Therefore, chitosan nano-particles provided sorption sites for neutral proteinase except the amino and hydroxyl groups.

![Fig.2 Infrared spectra of chitosan](image)

![Fig.3 Infrared spectra of chitosan nano-particles](image)

![Fig.4 Infrared spectra of chitosan nano-particles adsorbed neutral proteinase](image)

**Sorption kinetics for neutral proteinase**

*Influence of neutral proteinase concentration*

The different initial concentration of neutral proteinase solution having an effect on the sorption kinetics was illustrated in Fig. 5. Higher the initial concentration of neutral proteinase, it would take more time to reach equilibrium and had lower sorption rate. Neutral proteinase was adsorbed fast with more than 50% in 10 min. When the initial concentration of neutral proteinase was 1.0 mg/ml, the sorption rate reached 79.0% at 10 min. And the equilibrium time was 25 min. When the initial concentration of neutral proteinase was increased to 5.0 mg/ml, the sorption rate was 87.0% at 10 min. The sorption reached equilibrium after 25 min. And the sorption rate increased to 99%. Chitosan nano-particles could adsorb neutral proteinase quickly, and exhibit a high sorption capacity.

![Fig.5 Effect of neutral proteinase concentration on adsorption](image)

*Influence of nano-particles concentration*

With increase of nano-particles amount the amount of neutral proteinase adsorbed by chitosan nano-particles was increased. Fig. 6 shows the results obtained for 0.2, 0.3 and 0.5 mg of chitosan nano-particles (mean size 60 nm) in contact with 1.0 ml neutral proteinase solution.

![Fig.6 Effect of nano-particles concentration on adsorption](image)

*Influence of temperature*

Fig. 7 shows the sorption kinetics obtained at differing temperatures with chitosan nano-particles. The residual neutral proteinase concentration slightly increased with the increase of temperature from 25 to 45 °C. The sorption rate began to decrease at 45 °C. This phenomenon might be explained that chitosan nano-particles were assembled at high temperatures. Previous studies had shown the little differences for usual temperatures (in the range 5–55 °C) for copper, zinc or mercury sorption (20).
Influence of mean size

In Fig. 8, it could be seen higher sorption capacity when the mean size of chitosan nano-particles decreased. Chitosan nano-particles with mean size 60 nm had a high sorption capacity when they were contacted with 3.0 mg/ml neutral proteinase solution. Increasing the size of the nano-particles, the time required to reach equilibrium was increased. The contact surface of little size nano-particles could explain the differences among various mean sizes of nano-particles for neutral proteinase sorption. The sorption performance for chitosan and derivatives could be affected significantly by the particle size and the conditioning of the absorbent due to the diffusion restrictions caused by the low porosity and crystallinity of the raw chitosan (11). Chitosan nano-particles could reduce the crystallinity of the biopolymer and neutral proteinase to the expansion of the polymer network so as to increase the accessibility to internal sites and the sorption rates.

Influence of agitation speed

Fig. 9 shows that the sorption rate of neutral proteinase was increased significantly with increase of the agitation speed in short contact time, while the sorption rate showed little difference at equilibrium. Therefore, neutral proteinase sorption rate was independent of the agitation speed. Similar results for cadmium sorption on chitosan were obtained by Erosa et al. (9).

Sorption isotherms

Fig. 11 shows the experimental equilibrium isotherms for sorption of neutral proteinase on chitosan nano-particles. Neutral proteinase was absorbed very quickly within 25 min. After 25 min, the adsorption reached to equilibrate.

The first-order rate expression of Lagergren was given as:

\[
\log(q_e - q) = \log(q_e) - k_1 t / 2.3
\]
where \( q_e \) and \( q \) were the mounts of neutral proteinase adsorbed on chitosan nano-particles at equilibrium and at time \( t \), respectively, and \( k_1 \) was the rate constant of first-order adsorption. The slope and intercepts of the plots of \( \log(q_e - q) \) Vs \( t \) were used to determined the first-order rate constant \( k_1 \). The adsorption of neutral proteinase was fit to first-order of lagergren, \( k_1 \) was calculated was 23.65 h\(^{-1}\).

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The most important model of monolayer adsorption came from the work of Langmuir (17). Their sorption behaviors could be described with the Langmuir adsorption equation (3) as:

\[
\frac{C_e}{Q} = \frac{C_e}{Q_{\text{max}}} + \frac{1}{Q_{\text{max}}} b
\]

where \( C_e \) was the equilibrium concentration of neutral proteinase (mg/l), \( Q \) the amount of neutral proteinase adsorbed per unit weight of chitosan nano-particles of chitosan at equilibrium (mg/mg), \( Q_{\text{max}} \) the maximum sorption at monolayer coverage (mg/mg) and \( b \) was the Langmuir sorption equilibrium constant (ml/mg) and it was a measure of the energy of sorption.

A linearized plot of \( C_e/Q \) versus \( C_e \) (Fig. 12) was given \( Q_{\text{max}} \) and \( b \), the results obtained were: \( Q_{\text{max}} = 2.56 \text{ mg/mg} \) and \( b = 0.0013 \text{ (ml/mg)} \). The equation is \( C_e/Q = 0.3907C_e +0.0033. \) The plots demonstrated that the Langmuir equation provided a reasonable description of the experimental data.

The other well-known isotherm used to describe adsorption behavior was the Freundlich isotherm. The isotherm was another form of the Langmuir approach for adsorption on a heterogeneous surface. The amount of adsorbed material was the summation of adsorption on all sites. The Freundlich isotherm described reversible adsorption and was not restricted to the formation of the monolayer. This empirical equation took the form:

\[
C = K_F (Q_e)^{1/n}
\]

where \( K_F \) and \( n \) were the Freundlich constants characteristics of the system, \( K_F \) and \( n \) was indicator of the adsorption capacity and adsorption intensity, respectively. The slope and the intercept of the linear Freundlich equation were equal to \( 1/n \) and \( \log K_F \), respectively. In this experiment, \( n \) was 0.11, the adsorption intensity to neutral proteinase was weak.

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**Fig. 11** Adsorption isotherm

**Fig. 12** Langmuir adsorption isotherm

**Fig. 13** Freundlich adsorption isotherm

**Hypothesis of adsorption mechanism**

Neutral proteinase was cation when the initial solution pH was 7.0, chitosan nano-particles was anion.
The n is less than 0.5 in the Freundlich equation. We could know the absorption of neutral proteinase with chitosan nanoparticles was very easy. The interactivity strength between NH2 group, hydroxyl group and neutral proteinase was hydrogen bond force.

The hydrogen in the carboxyl group was connected with electronegative oxygen. Electron pair was attractive to oxygen, so hydrogen atom became cation and proton. When electronegative neutral proteinase was closed to chitosan nano-particles, the hydrogen bond might be formed. It was the main force between hydroxyl group, NH2 group and neutral proteinase.

Conclusions
Chitosan nano-particles have been prepared by ions gelation of chitosan and tri-polyphosphate. FT-IR spectra revealed the functional groups of chitosan nano-particles and the interaction with neutral proteinase, the amine and hydroxyl group of nanoparticles provided sorption sites for neutral proteinase. The experiments results showed that chitosan nano-particles could adsorb neutral proteinase from aqueous solution effectively. The experimental data of the sorption equilibrium from neutral proteinase solution correlated well with the Langmuir isotherm equation. The high sorption capacity of chitosan nano-particles for neutral proteinase indicated a promising adsorbent.

REFERENCES