Antibacterial activity of chitosan triplyphosphate nanoparticles loaded with various metal ions

Wen-Li Du a, Shan-Shan Niu b, Ying-Lei Xu a, Zi-Rong Xu a,* , Cheng-Li Fan a

a Institute of Feed Science, College of Animal Science, Zhejiang University, Key Laboratory of Molecular Animal Nutrition, Ministry of Education, Hangzhou 310029, China
b Laboratory of Fruit Molecular Physiology and Biotechnology, The State Agricultural Ministry Laboratory of Horticultural Plant Growth Development and Biotechnology, Department of Horticulture, Zhejiang University, Hangzhou 310029, China

ARTICLE INFO

Article history:
Received 19 January 2008
Received in revised form 26 July 2008
Accepted 31 July 2008
Available online 20 August 2008

Keywords:
Chitosan nanoparticles
Antibacterial activity
Particle size
Zeta potential

ABSTRACT

The aim of this study was to prepare and select chitosan nanoparticles loaded metal ions with high antibacterial activities. Chitosan nanoparticles were prepared based on ionic gelation between chitosan and sodium tripolyphosphate. Then, Ag⁺, Cu²⁺, Zn²⁺, Mn²⁺, or Fe²⁺ was individually loaded onto chitosan nanoparticles. Their particle sizes and zeta potentials were measured. Their antibacterial activities were evaluated by determination of minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) against Escherichia coli 25922, Salmonella choleraesuis ATCC 50020 and Staphylococcus aureus 25923 in vitro. Results showed that antibacterial activity was significantly enhanced by the metal ions loaded, except for Fe²⁺. Especially for chitosan nanoparticles loaded Cu²⁺, the MIC and MBC against E. coli 25922, S. choleraesuis ATCC 50020 and S. aureus 25923 were 21–42 times lower than that of Cu²⁺, respectively. Moreover, it was found that antibacterial activity was directly proportional to zeta potential.

1. Introduction

Chitosan has attracted considerable interest because of their unique combination of properties, such as biocompatibility, biodegradability, metal complexation and additional applications in various fields, for example, biotechnology (Mao et al., 2001), pharmaceutics (Illum, 1998), wastewater treatment (Rammnani & Sabharwal, 2006), cosmetics (Kumar, Muzzarelli, Muzzarelli, Sahiwa, & Domb, 2004), and food science (Chien, Seu, & Yang, 2007).

The antibacterial activity of chitosan has been widely explored (Hong, Park, Lee, & Meyers, 2002; Liu et al., 2006; Tsai, Zhang, & Shieh, 2004). A number of chitosan derivatives with different modifications have been prepared to improve its antibacterial activity (Jia, Shen, & Xu, 2001; Liu, Du, Yang, & Zhu, 2004; Yang, Chou, & Li, 2005). The metal-chelating property of chitosan has been mainly used in wastewater treatment. Recently, different metal-chitosan complexes have been prepared to improve its antimicrobial activity, for example, chitosan–Ag⁺ complex (Chen, Wu, & Zeng, 2005; Yi, Wang, & Liu, 2003) and chitosan–Zn²⁺ complex (Wang, Du, & Liu, 2004). Cu²⁺ exhibits high antibacterial activity in vitro (Domek, LeChevallier, & McFeters, 1984). Various kinds of Cu²⁺-organic complexes with remarkable antimicrobial activity have been synthesized (Carcelli, Mazza, Pelizzi, & Pelizzi, 1995; Jantova, Labuda, Vollek, & Zaskova, 1997).

Nanoparticles are made of natural or artificial polymers ranging in size from 10–1000 nm (Kreuter, 2001). Nanoparticles display unique physical and chemical features because of effects such as the quantum size effect, mini size effect, surface effect and macro-quantum tunnel effect. Chitosan triplyphosphate nanoparticles have been synthesized and mainly used as drug carrier as reported in previous studies (De Campos, Sánchez, & Alonso, 2001; Janes, Fresneau, Marazuela, Fabra, & Alonso, 2001; Xu & Du, 2003).

In our previous study, chitosan nanoparticles had been prepared, characterized, and used to adsorb eosin Y from aqueous solutions (Du, Xu, Han, Xu, & Miao, 2008). Here, Ag⁺, Cu²⁺, Zn²⁺, Mn²⁺ or Fe²⁺ was separately loaded onto chitosan nanoparticles. Then, particle size and zeta potential of the chitosan nanoparticle loaded with different metal ions were measured. In order to evaluate and compare their antibacterial activities, Escherichia coli 25922, Salmonella choleraesuis ATCC 50020 and Staphylococcus aureus 25923 were chosen as tested bacteria. Minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) of the materials against these bacteria were determined in vitro.

2. Materials and methods

2.1. Materials

Chitosan, originated from shrimp shell, was purchased from Yuhuan Ocean Biochemical Co. (Zhejiang Yuhuan, China). The degree of deacetylation and the molecular weight were about 90%...
and 150 kDa as determined by elemental analysis and viscometric method, respectively. Sodium tripolyphosphate (TPP) and chlortetracycline were obtained from Sigma Chemical Co., USA. The water used throughout this work was the reagent-grade water produced by Milli-Q SP ultra-pure-water system of Nihon Millipore Ltd., Tokyo. The other chemicals used were analytical grade reagents commercially available and used without further purification.

2.2. Preparation of the nanoparticles

Chitosan nanoparticles were prepared based on the ionotropic gelation between chitosan and sodium tripolyphosphate. Briefly, chitosan was dissolved in 1% (v/v) acetic acid to obtain a 0.3% (w/v) chitosan solution. TPP was dissolved in water to a concentration of 1%. Under magnetic stirring at room temperature, 1 ml of tripolyphosphate solution was added into 25 ml of chitosan solution. The mixture was stirred for 20 min, then, treated with sonication at 1.5 kW for 30 min. The suspension was subsequently centrifuged at 12,000g for 10 min. The precipitate was suspended in water, centrifuged again, then freeze dried. The freeze-dried chitosan nanoparticles were suspended in water for characterization or directly used for other experiments. Chitosan nanoparticles loaded Ag⁺, Cu²⁺, Zn²⁺, Mn²⁺, and Fe²⁺ were obtained by adding metal ion solutions into the chitosan nanosuspensions (0.3%, w/v) to reach a final concentration of 120 µg/ml and stirring for 12 h at room temperature. Chitosan nanoparticles loaded with metal ions were further purified as described above.

2.3. Characterizations

Particle size and zeta potential were measured using a Zetasizer Nano-ZS-90 (Malvern Instruments). The analysis was performed at a scattering angle of 90° under 25 °C. For zeta potential measurements, samples were dispersed in 0.1 mM KCl and measured under the automatic mode. For chitosan nanoparticles loaded Ag⁺, 0.1 mM KNO₃ was used to replace KCl in order to avoid the reaction between Ag⁺ and Cl⁻.

![Fig. 1. Size distribution. (A) Chitosan nanoparticles; (B) chitosan–Ag nanoparticles; (C) chitosan–Cu nanoparticles; (D) chitosan–Zn nanoparticles; (E) chitosan–Mn nanoparticles; (F) chitosan–Fe nanoparticles.](image)
2.4. Bacteria growth conditions

*Escherichia coli* 25922, *S. choleraesuis* ATCC 50020 and *S. aureus* 25923, provided by the Center for Typical Culture Collection of China, were used to evaluate the antibacterial activity. Muller–Hinton (MH) broth and MH agar (Difco, USA) were used as growth media.

Bacteria were incubated at 37 °C shaken in a thermostat. At the exponential phase, bacteria were harvested by centrifuge at 4000 g for 10 min under 4 °C, then, washed twice with 10 mM phosphate buffer saline (PBS, pH 7.2). The bacteria were suspended in PBS and adjusted to \(1 \times 10^7\) CFU/ml for further use.

2.5. Evaluation of antibacterial activity in vitro

The minimum inhibitory concentration was determined by a broth dilution method, recommended by the NCCLS (NCCLS, 2000). The chitosan, chitosan nanoparticles, chitosan nanoparticles loaded different metal ions, silver nitrate, copper sulfate, zinc sulfate, manganese sulfate, ferric sulfate and chlortetracycline were gradually diluted in MH broth. Chitosan was also diluted in MH broth, which contained 0.25% (v/v) acetic acid due to its insolubility. Bacteria were inoculated to achieve a bacterial concentration of \(1 \times 10^5\) CFU/ml. MIC was read after 24 h of incubation at 37 °C equivalent to the concentration of the tube without visible growth. To evaluate MBC, a sample of 100 μl was transferred from each tube without visible growth to a MH agar plate and incubated at 37 °C for another 24 h. The MBC was read as the concentration of the tube without bacterial growth. The test was performed triplicate for each bacterium. Value that agreed on two or more occasions was adopted as the MIC or MBC of the strain.

3. Results and discussion

3.1. Particle size and zeta potential

Size (including size distribution) and zeta potential are essential characteristic parameters for nanosuspensions (Müller, Jacobs, & Kayser, 2001). Fig. 1 showed size distribution profiles of the chito-
san nanoparticles and nanoparticles loaded different metal ions. Chitosan nanoparticles had a mean diameter of 53.99 nm with a narrow size distribution (Width: 5.359 nm; Polydispersity index: 1.000) as shown in Fig. 1A. Both the mean diameter and the size distribution increased when metal ions were loaded (Fig. 1B–F). The mean diameters of chitosan nanoparticles, loaded Ag⁺, Cu²⁺, Zn²⁺, Mn²⁺, or Fe²⁺, were 90.29, 121.9, 210.9, 102.3, and 95.81 nm, respectively.

As shown in Fig. 2, the chitosan nanoparticles had a zeta potential of +51.37 mV (Fig. 2A). The zeta potentials were enhanced significantly due to the loading of metal ions. The reason probably resulted from the positive charge carried by metal ions, which were loaded onto chitosan nanoparticles. The zeta potential of the nanoparticles loaded Ag⁺, with a +92.05 mV, was the highest, following by Cu²⁺ loaded, which was +88.69 mV. The zeta potentials of nanoparticles loaded Zn²⁺ and Mn²⁺ were +86.65 and +75.74 mV, respectively. The nanoparticles loaded Fe²⁺ had the lowest zeta potential of +71.42 mV, but still higher than that of the chitosan nanoparticles. Zeta potential is a crucial parameter for stability in aqueous suspensions. For a physically stable nanosuspension solely stabilized by electrostatic repulsion, a zeta potential of ±30 mV is required as a minimum (Müller et al., 2001). All these data suggested that chitosan nanoparticles and chitosan nanoparticles loaded metal ions prepared here were stable.

3.2. Antibacterial activity

The MIC and MBC values against E. coli, S. choleraesuis, and S. aureus are shown in Table 1. Antibacterial activity was directly proportional to zeta potential. Generally, antibacterial activity was significantly enhanced by metal ion loaded, especially for Cu²⁺ and Zn²⁺, compared to those of chitosan nanoparticles and metal ions. It was found that antibacterial activity was directly proportional to zeta potential. Moreover, Gram-negative bacteria were more sensitive than Gram-positive bacteria.

### Table 1

<table>
<thead>
<tr>
<th>Sample</th>
<th>E. coli</th>
<th>S. choleraesuis</th>
<th>S. aureus</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>MIC</td>
<td>MBC</td>
<td>MIC</td>
</tr>
<tr>
<td>Chitosan</td>
<td>–</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>Chitosan nanoparticles</td>
<td>468</td>
<td>750</td>
<td>468</td>
</tr>
<tr>
<td>Chitosan nanoparticles loaded Ag⁺</td>
<td>117</td>
<td>187</td>
<td>117</td>
</tr>
<tr>
<td>Chitosan nanoparticles loaded Cu²⁺</td>
<td>9</td>
<td>12</td>
<td>9</td>
</tr>
<tr>
<td>Chitosan nanoparticles loaded Zn²⁺</td>
<td>18</td>
<td>24</td>
<td>18</td>
</tr>
<tr>
<td>Chitosan nanoparticles loaded Mn²⁺</td>
<td>73</td>
<td>97</td>
<td>73</td>
</tr>
<tr>
<td>Chitosan nanoparticles loaded Fe²⁺</td>
<td>121</td>
<td>195</td>
<td>121</td>
</tr>
<tr>
<td>Ag²⁺</td>
<td>4</td>
<td>8</td>
<td>4</td>
</tr>
<tr>
<td>Cu²⁺</td>
<td>256</td>
<td>512</td>
<td>256</td>
</tr>
<tr>
<td>Zn²⁺</td>
<td>768</td>
<td>1024</td>
<td>768</td>
</tr>
<tr>
<td>Mn²⁺</td>
<td>1472</td>
<td>1536</td>
<td>1472</td>
</tr>
<tr>
<td>Fe²⁺</td>
<td>1728</td>
<td>1856</td>
<td>1728</td>
</tr>
</tbody>
</table>

Table 1: MIC and MBC values against E. coli, S. choleraesuis, and S. aureus (μg/ml).

**References**


Zhang, Jiang, Ding, Malcolm, and David (2007) reported that the antibacterial activity of ZnO nanoparticles increased with decreasing particle size. But, our data showed that the zeta potential influence antibacterial activity of the nanoparticles much more, while particle size throw little effect on antibacterial activity, if any.

4. Conclusions

Generally, antibacterial activity was significantly enhanced by metal ion loaded, especially for Cu²⁺ and Zn²⁺, compared to those of chitosan nanoparticles and related metal ions. It was found that antibacterial activity was directly proportional to zeta potential. Moreover, Gram-negative bacteria were more sensitive than Gram-positive bacteria.


