Carbohydrate Polymers 155 (2017) 192–200

Contents lists available at ScienceDirect

Carbohydrate Polymers

journal homepage: www.elsevier.com/locate/carbpol

Integration of lysozyme into chitosan nanoparticles for improving antibacterial activity

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ARTICLE INFO

Article history:
Received 14 May 2016
Received in revised form 19 August 2016
Accepted 25 August 2016
Available online 25 August 2016

Keywords:
Chitosan nanoparticles
Chitosan-lysozyme nanoparticles
Sodium tripolyphosphate
Ionotropic gelation
Antibacterial activity

ABSTRACT

Lysozyme was integrated into chitosan nanoparticles (CS-NPs) to improve the antibacterial activity. CS-NPs and chitosan-lysozyme nanoparticles (CS-Lys-NPs) were prepared according to the ionic gelation technique and then characterized by average size, zeta potential, polydispersity index (PDI), atomic force microscopy (AFM), fourier transform infrared (FT-IR), small-angle X-ray scattering (SAXS), circular dichroism (CD) and UV–visible spectroscopy. Antibacterial properties were investigated by transmission electron microscopy (TEM) based on observation of the inhibition zone and measurement of the minimum inhibitory concentration (MIC) and minimum bacterial concentration (MBC) of CS-NPs and CS-Lys-NPs against *E. coli and B. subtilis*. The CS-NPs had particle sizes of 476.2–548.1 nm, while an increase to ~488.8 to 613.5 nm was observed upon loading with lysozyme. The results suggested that the integration of lysozyme into CS-NPs enhanced the antibacterial activity against *E. coli* and *B. subtilis*, which may show great potential for use in the food industry and other applications in the form of direct addition or incorporation into packaging.

1. Introduction

Lysozyme is a small monomeric protein. It is a glycoside hydrolase composed of 129 amino acid residues, which contains four intact disulfide bonds, six tryptophan (Trp), three tyrosine (Tyr), three phenylalanine (Phe) residues (Jing, Chang et al., 2016; Jing, Song et al., 2016). lysozyme ubiquitously presents in various human tissues and secretions (Chen et al., 2005).The unique characteristic of anti-inflammatory, antiviral, antiseptic, antihistamine and antineoplastic activities make it has potential in pharmacological functions (Jash & Kumar, 2014). Lysozyme has been applied in the preservation of different food products (e.g. rawand processed meat, dairy products, fruits and vegetables) to extend the shelf life (Tiwari et al., 2009). Additionally, various applications of lysozyme were developed, such as the potential to be an antimicrobial agent in pharmaceuticals, home appliances and potential aseptic and therapeutic uses, which make lysozyme applications an interesting research area (Zheng, Wan, Yu, & Zhang, 2016).

It is well known that lysozyme can inhibit some gram positive bacteria for its unique ability to damage bacterial by hydrolyzing 1,4-β-linkage between N-acetyl- muramic acid and N-acetyl-D-glucosamine of bacterial cell wall peptidoglycan (Jollès, 1964; Yuan, Yin, Jiang, & Liang, 2013). However, gram negative bacteria are less sensitive toward lysozyme due to the presence of a protective lipopolysaccharide (LPS) on the bacterial cell wall. What is more, the practical application of free lysozyme is quite limited because this molecule is unstable and easily inactivated (Zhang et al., 2015).

To improve the quality of lysozyme, many methods were developed. Lysozyme combined with other antimicrobial agents exihibited a better antimicrobial activity (Chen et al., 2005). Besides, lysozyme was prepared in the forms of microcapsules, films, beads, nanoparticles etc. incorporated with other materials (Amara, Eghbal, Degraeve, & Gharsallaoui, 2016; Dekina, Romanovska, Ovsepyan, Tkach, & Muratov, 2016; Liburdi, Benucci, Palumbo, & Esti, 2015; Jing, Chang et al., 2016; Jing, Song et al., 2016). Wherein, protein/polysaccharide complexes are important for industrial applications. Sugars like sucrose can protect lysozyme structure by forming hydrogen bonds with protein molecules (Liao,

Abbreviations: CS, chitosan; CS, NPs chitosan nanoparticles; CS-Lys-NPs, chitosan-lysozyme nanoparticles; TPP, sodium tripolyphosphate.

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http://dx.doi.org/10.1016/j.carbpol.2016.08.076
0144-8617/

Brown, Nazir, Quader, & Martin, 2002).The coating of lysozyme with poly-γ-glutamic acid and chitosan can broaden the antibacterial spectrum of the composite lysozyme nano reagent, and improve antibacterial activity (Yong et al., 2013).

Chitosan (CS) is a positively charged polysaccharide formed by deacetylation of chitin. As the second most abundant biopolymer, chitin is ubiquitous in the cell wall of fungi and living organisms such as shrimps, crabs, insects and tortoises (Ravindra, Krovvidi, & Khan, 1998). CS can be used in solutions, hydrogels, micro-particles and nanoparticles because of its unique versatility. In addition, an endless array of CS derivatives can be prepared because of the existing amine, N-acetyl and hydroxyl groups. CS is widely used in the delivery system of proteins, drugs, vaccines and enzymes because of its excellent mucoadhesive characteristics, which extend the residual time at the absorption site (Sanyakamdhorn, Agudelo, & Tajmir-Riahi, 2013).

For a delivery system, it is vital to control characteristics such as particle size, particle distribution and surface charge because these parameters affect the behavior of core materials. There are significant connections between particle size and the effects on antigen delivery to antigen-presenting cells. It was reported that antigen-presenting cells such as dendritic cells (DC) could uptake particles of 0.5 μm and smaller with the best performance (Foged, Brodin, Frokjaer, & Sundblad, 2015). Lately, chitosan-based nanoparticles (CSNPs) have exhibited various advantages over the pattern chitosan. The enhanced antimicrobial activity and controlled release rate made chitosan-based nanoparticles an ideal candidate for applications in targeted delivery systems in the food and pharmacy industries. Compared to CS, the nano size and larger surface area contribute to the improved interactions between CSNPs and the microbial cell wall. The larger surface area allows nanoparticles to attach tightly onto the surface of bacteria, which damages the intracellular components and accelerates the death of cells, resulting in better antibacterial activities of CSNPs (Qi, Xu, Jiang, Hu, & Zou, 2004).

In the present study, CS nanoparticles were prepared, and lysozyme was integrated into CS-NPs based on the ionic gelation of chitosan with tripolyphosphate anions. Their antibacterial activities against gram negative bacteria *Escherichia coli* O_{157}:H_7 (ATCC25922) (*E. coli*) and gram positive bacteria *Bacillus subtilis* (ACCC10242) (*B. subtilis*) were examined. The morphological changes of *E. coli*/*B. subtilis* treated with CS, CS-NPs and CS-Lys-NPs were examined by transmission electron microscopy (TEM). And the secondary structure of lysozyme and its possibly interactions between chitosan particles were studied, which could contribute to the antimicrobial actions.

2. Materials and methods

2.1. Reagents and bacteria

Chitosan (molecular weight of 50,000 to 100,000) was purchased from Qingdao Yun Yu Biotechnology Co., Ltd. Lysozyme (from chicken egg white) and sodium tripolyphosphate (TPP) were purchased from Sigma Chemical Co. (St. Louis, MO, USA). Nutrient Broth (NB) and Nutrient Agar (NA) were purchased from Qingdao Hope Bio-Technology Co., Ltd. Acetic acid, sodium hydroxide, sodium dihydrogen phosphate, disodium hydrogen phosphate were of analytical grade (AR), and hydrochloric acid was of guarantee reagent (GR), all of these were purchased from Sinopharm Chemical Reagent Co., Ltd. *Bacillus subtilis* ACCC10242 (*B. subtilis*) was obtained from Life sciences insititute, Zhejiang university, China. *Escherichia coli* O_{157}:H_7 (ATCC25922) (*E.coli*) was purchased from China center of industrial culture collection (CICC).

2.2. Preparation of chitosan nanoparticles

Chitosan nanoparticles were prepared based on the ionotropic gelation between CS and sodium tripolyphosphate (TPP) as reported previously (Jang & Lee, 2008) with slight modifications. Briefly, solutions with CS-to-TPP ratios of 3:1 (V/V) were prepared and stirred at room temperature (25 ± 1 °C) to achieve complete dissolution, adjusting the final pH value to 4 and 5 with hydrochloric acid (1 M) and sodium hydroxide (1 M). CS-NPs were formed by the addition of 6 mL TPP (0.25%, W/V) solution using a peristaltic pump (BT100-2J, Hebei Lange current pump Ltd, China) to 18 mL CS solution (0.50%, W/V) under constant magnetic stirring for 1 h. Nanoparticles were purified by centrifugation at 10000 × *g* for 15 min. The precipitate was extensively rinsed with distilled water to remove unreacted substance and then freeze-dried with a freeze dryer (Labconco, Beijing Light Ace HK L-imited, China) for 2 days under −80 °C before further use or analysis.

Lysozyme was integrated into 10 mL CS nanoparticle suspensions (0.5% w/v) by adding lysozyme at five different concentrations, 0.25, 0.50, 0.75, 1.00 and 1.25 mg/mL, before purification. The purification was then carried out as described above for the CS-NPs. The pHs used were based on the antimicrobial actions according to previously studies (Hong, Na, Lee, & Meyers, 2002; Zhang et al., 2016) and preliminary analysis.

2.3. Characterization of CS-Lys-NPs

CS-NPs prepared at pH 5 and CS-Lys-NPs prepared at pH 5 with lysozyme concentrations of 1.25 mg/mL were characterized for most measurements based on the preliminary analysis of encapsulation efficiency, polydispersity index.

The particle size, polydispersity index (PDI) and zeta potential (ZP) of the resultant particles were determined by nano particle size and zeta potential analyzer (Zetasizer Nano ZS90, UK).

The surface micrographs were imaged by atomic force microscopy (AFM, Multimode, BRUKER Daltonics Inc., USA). CS, CS-NPs and CS-Lys-NPs solutions were added to the surface of a piece of mica plate (10 × 10 mm, Beijing Zhongjingkeyi Technology Co., Ltd, China) and dried naturally after 24 h before AFM observation.

Fourier transform infrared (FT-IR) spectroscopy (Thermo Scientific Nicolet Avatar 370, USA) was carried out to confirm information about chemical bonds or functional groups, with the spectrum collected from 4000 to 400 cm^{-1} at room temperature at 4 cm^{-1} resolution.

Diffraction patterns of small-angle X-ray scattering (SAXS) were obtained using a diffractometer (Xenocs-3D SAXS, France). CS-NPS and CS-Lys-NPs solutions were purified by centrifugation, and then the washed precipitates were scanned with 10 min of exposure time at room temperature.

The UV analyses for CS-NPs and CS-lysozyme nanoparticles were performed on a UV–visible spectrophotometer (UV-2550, Japan). Samples at 0.1 mg/mL were dissolved in an acetic acid buffer (pH 5.0) for measurement. One curve was obtained after three scans.

Circular dichroism analysis was carried out to confirm the secondary structure of lysozyme with a Multifunctional CD Spectroscope (Francois Goy, MOS-450). Samples were adjusted to a concentration of 0.1 mg/mL in an aqueous buffer (pH 5.0). One curve was obtained after three scans.

2.4. Measurement of inhibition zones, minimum inhibitory concentration (MIC) and minimum bacterial concentration (MBC)

Gram positive bacteria *B. subtilis* and gram negative bacteria *E. coli* were chosen to determine the antimicrobial actions of the