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3,6-*O*-[*N*-(2-Aminoethyl)-acetamide-yl]-chitosan exerts antibacterial activity by a membrane damage mechanism

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ABSTRACT

A novel chitosan derivative, 3,6-*O*-[*N*-(2-aminoethyl)-acetamide-yl]-chitosan (AACS), was successfully prepared to improve water solubility and antibacterial activity of chitosan. AACS had good antibacterial activity, with minimum inhibitory concentrations of 0.25 mg/mL, against *Escherichia coli* and *Staphylococcus aureus*. Cell membrane integrity, electric conductivity and NPN uptake tests showed that AACS caused quickly increasing the release of intracellular nucleic acids, the uptake of NPN, and the electric conductivity by damaging membrane integrity. On the other hand, hydrophobicity, cell viability and SDS-PAGE experiments indicated that AACS was able to reduce the surface hydrophobicity, the cell viability and the intracellular proteins through increasing membrane permeability. SEM observation further confirmed that AACS could kill bacteria via disrupting their membranes. All results above verified that AACS mainly exerted antibacterial activity by a membrane damage mechanism, and it was expected to be a new food preservative.

1. Introduction

Antibacterial agents have become indispensable for the treatment of widespread bacterial diseases in many fields, such as medical and health, agriculture, and food industry. Illness caused by intake of food contaminated with pathogenic bacteria or their toxins has been of vital concern (Lee & Je, 2013). Traditional chemical fungicides and pesticide residues are usually harmful to human body, and many of them are not biodegradable or environment-compatible (Gabriel, Tiera, & Tiera, 2015), so that developing new antibacterial materials, which can prevent or inhibit the growth and reproduction of pathogens, has become an urgent issue (Nathan, 2004; Tang, Shi, Zhao, Hao, & Le, 2009).

The search for more environment-friendly antibacterial materials has promoted research on natural materials, such as essential oils (Gabriel et al., 2015), polysaccharides (Gabriel et al., 2015) and antimicrobial peptides (Tang et al., 2009). Among them, chitosan (CS) has emerged as a promising and economic source for versatile materials (Tang et al., 2009). It has many excellent biological

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properties such as biocompatibility, biodegradability, hemostatic activity, non-antigenic property, antibacterial activity, and wound-healing activity (Burkatovskaya et al., 2006; Sashiwa & Aiba, 2004). One of the noticeable properties is antibacterial activity, particularly against Gram-negative and Gram-positive bacteria (Belalia, Grelier, Benaissa, & Coma, 2008; Rabea, Badawy, Stevens, Smagghe, & Steurbaut, 2003). Since the good antibacterial activity and broad spectrum, CS is getting more popular in food preservation (Huang et al., 2012).The potential of CS to act as a food preservative has been widely reported on the basis of *in vitro* trials as well as through direct application on real complex matrix foods (Vásconez, Flores, Campos, Alvarado, & Gerschenson, 2009). However, CS has a few disadvantages, especially the poor solubility, which limits its further application in antibacterial field. Many CS derivatives, such as chitosan-thioglycolic acid (Geisberger et al., 2013), *O*-acetyl-chitosan-*N*-2-hydroxypropyl trimethyl ammonium chloride (Cai et al., 2015),

quaternized chitosan (Peng et al., 2010), 6-amino-6-deoxy-chitosan (Satoh et al., 2006), chitosan-arginine (Tang et al., 2010), and aminoethyl-chitosan (Je & Kim, 2006), have been prepared to improve water solubility and antibacterial activity of CS. Yet the CS derivatives (Geisberger et al., 2013; Peng et al., 2010) with quaternary ammonium and mercapto groups exhibit high toxicity to human body. Comparatively, a few CS derivatives (Je & Kim, 2006; Satoh et al., 2006; Tang et al., 2010) with more NH_2 groups show low toxicity and are preferable for use in medical and health or food field, so it has aroused much attention to introduce more NH_2 groups into CS.

Since some CS derivatives are designed as effective antibacterial agents, it is essential to investigate the antibacterial mechanism of CS. Several main speculative antibacterial mechanisms of CS have been proposed: (1) electrostatic interactions between the cationic amino groups of CS and the anionic substances (*e.g.* phospholipid and teichoic acid) on bacterial surface, which would lead the cell wall and plasma membrane perturbation (Zakrzewska et al., 2007) and the subsequent leakage of intracellular content (Helander, Nurmiaho-Lassila, Ahvenainen, Rhoades, & Roller, 2001; Raafat, Von Bargen, Haas, & Sahl, 2008; Xing, Zhu, Peng, & Qin, 2015; Zakrzewska et al., 2007); (2) metal-chelation, which might disrupt the outer membrane (OM) of Gram-negative bacteria (Palma-Guerrero et al., 2010) or microbial cell wall; and (3) interactions between cellular nucleic acids and the internalized CS, which might interfere with gene expression (Tao, Qian, & Xie, 2011; Tan et al., 2012). The speculative mechanisms of antibacterial action of CS indicate the antibacterial activity is mainly caused by the free amino groups ($-NH_2$). Accordingly, to prepare CS derivatives with more $-NH_2$ groups benefits in improving the antibacterial activity, and further exploring the antibacterial mechanism of CS. Many efforts have been made to conjugate $-NH_2$ groups onto CS, however, the reaction conditions are usually critical, such as under an N_2 atmosphere (Satoh et al., 2006). It is thus significant to develop novel CS derivatives or new synthetic methods, in order to introduce more $-NH_2$ groups into CS.

This work was to prepare a new water-soluble CS derivative, study its antimicrobial activity, and reveal antibacterial mechanisms. 3,6-*O*-[*N*-(2-Aminoethyl)-acetamide-yl]-chitosan (AACS) was prepared by conjugating ethylenediamine onto *O*-carboxymethyl chitosan. Antibacterial activity assay was conducted via testing the minimum inhibitory concentration. The model of action of AACS on *Escherichia coli* (*E. coli*) and *Staphylococcus aureus* (*S. aureus*) was evaluated using cell membrane integrity assay, electric conductivity measurement, hydrophobicity test, NPN uptake, SDS-PAGE, cell viability assay, and scanning electron microscopy (SEM). Additionally, by all the tests above, this paper aimed to improve the property of CS, and verify the application feasibility of AACS as an antibacterial agent in food preservation.

2. Materials and methods

2.1. Materials

Chitosan (CS) extracted from *Chionoecetes bairdi* shells was purchased from Shanghai Lanji Science and Technology Development Co., Ltd. (China). EDC·HCl was purchased from Shanghai Yuanye Bio-Technology Co., Ltd. (China). *N*-Phenyl-1-naphthylamine (NPN), propidium iodide (PI) and HEPES sodium salt were obtained from Sigma Chemical Co. (St. Loius, MO). All other chemical reagents were of analytical grade, and were purchased from Sinopharm Chemical Reagent Co., Ltd. *E. coli* (ATCC 25992) and *S. aureus* (ATCC 25923) supplied by Microbiology Laboratory of Ocean University of China were used for antibacterial activity and antibacterial mechanism research of AACS.

2.2. Molecular weight and deacetylation degree of CS

The molecular weight of CS was determined using an Ubbelohde Viscometer at 25 °C. The deacetylation degree of CS was ascertained according to the method of acid-base titration. All these methods are traditional methods and have been described in our previously published literature (Liu et al., 2006).

2.3. Synthesis and characterization of AACS

The reaction scheme for the synthesis of AACS is shown in Fig. 1. The NH_2 groups of CS were protected with benzaldehyde referring to the relevant literature (Wang & Wang, 2011) with slight changes. CS (5 g) was dissolved in 1% (w/v) acetic acid prior to the addition of 100 mL of anhydrous ethanol, and then dropping benzaldehyde that dissolved in 30 mL anhydrous ethanol into the solution with stirring at 60 °C. After 5 h, the solution was poured into anhydrous ethanol to remove the extra benzaldehyde. Then the solution was poured into anhydrous ethanol to remove the extra benzaldehyde. The floccule (*N*-Benzylidene CS) was washed three times with anhydrous ethanol and dried at 50 °C for 24 h.

N-Benzylidene CS (5 g) was soaked in 30 mL of 50% (w/v) NaOH solution for 2 h prior to the addition of 50 mL of isopropanol. The mixture was set at −20 °C for several hours. After the mixture was thawed at 50 °C, 10 g of monochloroacetic acid was dissolved in 10 mL of isopropanol and then dropped into the mixture above within half an hour. The temperature was maintained at 50 °C for 6 h until the reaction was completed, and then the pH was adjusted to 7 at room temperature prior to filtering the mixture. The precipitate was soaked in 0.1 M HCl for 12 h, and then the mixture was poured into substantial anhydrous ethanol. The floccule, *O*-carboxymethyl chitosan (CMCS), was rinsed thoroughly with 75% ethanol, dewatered with anhydrous ethanol, and dried *in vacuo*.

CMCS (0.5 g) was dissolved in 20 mL of distilled water, and 1.0 mL of ethylenediamine was added prior to adjusting the pH value to 4–5 with 1.0 M HCl. EDC·HCl (0.2 g) was dissolved in 10 mL of deionized water and added dropwise to the CMCS solu-

Table 1
Minimum inhibitory concentrations of AACS and CS against *E. coli* and *S. aureus*.[a]

Strain	MIC (mg/mL)	
	CS	AACS
E. coli	0.375	0.25
S. aureus	0.25	0.25

[a] AACS: 3,6-*O*-[*N*-(2-aminoethyl)-acetamide-yl]-chitosan; *E. coli*: *Escherichia coli*; *S. aureus*: *Staphylococcus aureus*; MIC: minimum inhibitory concentration.