Carbohydrate Polymers 155 (2017) 321–328

Contents lists available at ScienceDirect

Carbohydrate Polymers

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

Synthesis, characterization, and antimicrobial activities of sulfonated chitosan

Zhimin Sun, Changgu Shi, Xiangyang Wang, Qiang Fang, Jianying Huang*

Key Laboratory of Fruits and Vegetables Postharvest and Processing Technology Research of Zhejiang Province, College of Food Science and Biotechnology, Zhejiang Gongshang University, Hangzhou 310018, PR China

ARTICLE INFO

Article history:
Received 3 July 2016
Received in revised form 16 August 2016
Accepted 20 August 2016
Available online 22 August 2016

Keywords:
Chitosan
Antimicrobial activity
Scanning electron microscopy
Arthrinium sacchari.

ABSTRACT

Sulfonated chitosan (SCS) was prepared via a facile chemical procedure by using 1,3-propane sultone attached to the backbone of chitosan. The resulting chitosan derivative was characterized by Fourier transform-infrared spectroscopy, nuclear magnetic resonance spectroscopy, gel permeation chromatography, elemental analysis, and thermo gravimetric analysis. The antibacterial experiments demonstrated that SCS exhibited higher antibacterial activities against *Escherichia coli* and *Staphylococcus aureus* with the minimum inhibitory concentration (MIC) of 0.13 mg/mL and 2.00 mg/mL than those of water-soluble chitosan (WCS) with MIC of 0.50 mg/mL and 4.00 mg/mL. However, different antifungal activities of SCS against *Arthrinium sacchari* (MIC, 64.00 mg/mL) and *Botrytis cinerea* (MIC, 0.25 mg/mL) were observed. Scanning electron microscopy revealed that WCS and SCS could damage and deform the structure of fungal hyphae with different action modes. It is worth mentioning that SCS exhibited selective antifungal activity, which was an important parameter to control its biological performance in many fields.

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1. Introduction

Chitosan, partially deacetylated derivative of chitin, is well known to be inexpensive, nontoxic, biocompatible and efficiently antimicrobial material (Meng, Yang, Kennedy, & Tian, 2010; Pillai, Paul, & Sharma, 2009). It has been widely utilized in biomedical, agricultural and food industrial. However, the remarkably poor solubility of chitosan due to the high crystallinity induced by hydrogen bonds and acetamido groups limits its application. Thus, it is necessary to modify the structure of chitosan to create derivatives with improved properties. Although chemical modification of chitosan to generate new bio-functional materials has achieved success (Geng, Yang, Huang, Zhang, & Wang, 2013; Jia, Duan, Fang, Wang, & Huang, 2016; Li, Duan, Huang, & Zheng, 2016; Zhang, Geng, Jiang, Li, & Huang, 2012), a strategy to reduce the positive charges of protonated amino groups presented on the polymeric surface, especially the introduction of sulfonate groups (RSO_3^-) on the chitosan chain, is still of great interest.

Anionic polysaccharides, in which their structures include sulfate, exhibit good biological activity and biocompatibility. For instance, heparin (HP), a linear biopolymer that contains sulfated *D*-glucosamine units, can be used to form polyelectrolyte complexes with cationic polymers at specific conditions of pH (Bueno et al., 2015; Fu, Ji, Yuan, & Shen, 2005; Martins, Pereira, Fajardo, Rubira, & Muniz, 2011) and act indirectly in the blood coagulation mechanism to stimulate cellular growth, as well as exhibit antibacterial effects (Bueno et al., 2015). Chondroitin sulfate (ChS), another polysaccharide containing sulfonate groups, is an ingredient in health foods for the treatment of symptoms related to arthritis and cartilage repair because negatively charged ChS is capable of electrostatic interaction with positively charged growth factors to stabilize and prevent degradation of the growth factors in medium (Cai, Liu, Shu, & Prestwich, 2005; Lim et al., 2011).

A few useful matrixes containing chitosan and different anionic natural polyelectrolytes, such as heparin (Liu, Jiao, Liu, & Zhang, 2007) and chondroitin sulfate (Yeh, Cheng, Hu, Huang, & Young, 2011) have been developed for biocompatible applications and environmentally sensitive bio-materials (Tsai et al., 2015). However, ChS–CS nanoparticles are expected to be formed under acidic conditions (pH below 6.5), limiting their applications in the delivery of acid-sensitive proteins and drugs. The presence of reactive hydroxyl and amino groups of chitosan enables physicochemical modifications to its structure thus obtaining versatile functional properties. Jayakumar, Nwe, Tokura, and Tamura (2007) have well reviewed sulfated chitin and chitosan and their synthetic methods and potential applications. For example, the introduction of

* Corresponding author.
E-mail address: huangjy@zjgsu.edu.cn (J. Huang).

http://dx.doi.org/10.1016/j.carbpol.2016.08.069
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sulfonate groups into chitosan chain could improve its biological and chemical properties, such as blood anticoagulant activity (Suwan et al., 2009), antioxidant activity (Huang, Mendis, & Kim, 2005), hemagglutination inhibition, adsorption of metal ions (Ruiz, Sastre, & Guibal, 2003), antimicrobial activity (Zhong, Li, Xing, & Liu, 2009), as well as anti HIV-1 activity (Nishimura et al., 1998). Sulfonated chitosan (0.63% sulfur content) exhibited antibacterial activity against *Shigella dysenteriae*, *Aeromonas hydrophila*, *Salmonella typhimurium*, *and Bacillus cereus* for the application in oyster preservation (Chen, Liau, & Tsai, 1998).

However, there still few information on the antimicrobial activities of SCS obtained by a facile chemical substitution with 1,3-propane sultone, including the antifungal activities against *A. sacchari* and *B. cinerea*. The objective of this study is to introduce sulfate group to the backbone of chitosan and to investigate the antimicrobial activity of the resulting anionic chitosan. Herein, sulfonic group was introduced into chitosan by nucleophilic substitution to afford *N*-(3-sulfonic) chitosan (sulfonated chitosan, SCS). The resulting anionic materials were characterized by Fourier transform-infrared spectroscopy (FT-IR), ^{1}H and ^{13}C nuclear magnetic resonance (NMR) spectroscopy, gel permeation chromatography (GPC), elemental analysis (EA), and thermo gravimetric analysis (TGA). Antimicrobial activities of sulfonated chitosan against *E. coli*, *S. aureus*, and *A. sacchari*, *B. cinerea* were further assessed and compared with that of water-soluble chitosan, followed the morphological hyphae changes investigation using scanning electron microscopy (SEM) techniques.

2. Materials and methods

2.1. Materials

The chitosan (CS) with the deacetylation of 91% and a centipoise viscosity of 60 mPa s (1%, 20 °C) and water-soluble chitosan (WCS, water-soluble chitosan hydrochloride) with the deacetylation of 92% and a centipoise viscosity of 22 mPa s (1%, 20 °C) used in the experiments were purchased from Qingdao Honghai Biotechnology Company (Shandong, China). Nutrient broth (NB), plate count agar (PCA), potato dextrose broth (PDB), and potato dextrose agar (PDA) were purchased from Hangzhou Microbial Reagent Co., Ltd. (Zhejiang, China). Other reagents and solvents were purchased from Aladin Reagent Co., Ltd. (Shanghai, China) and were used directly without further purification.

2.2. Microorganisms

Escherichia coli (*E. coli*, CICC 21524) and *Staphylococcus aureus* (*S. aureus*, CICC 10384) used in this study were purchased from China Center of Industrial Culture Collection (Beijing, China). *Botryis Cinerea* (*B. cinerea*, 3.611) was purchased from Beijing Yudingxinjie Technology Co., Ltd. (Beijing, China). *Arthrinium sacchari* (*A. saccahri*, KC354569) strain was isolated from deteriorated blueberries and identified by Sangon Biotech Co., Ltd. (Shanghai, China).

2.3. Synthesis of sulfonated chitosan (SCS)

As shown in Scheme 1, SCS was prepared by modifying CS using 1,3-propane sultone according to the method described by Rwei and Lien (Rwei & Lien, 2014) with minor modification. One gram of CS was completely dissolved in 80 mL of 2 wt% aqueous acetic acid solution. Afterwards, 2 g of 1,3-propane sultone was added dropwise into CS solution under nitrogen atmosphere, and the result mixture was stirred at 60 °C for 6 h. The solute was then poured into cold acetone with vigorous stirring to precipitate the white product. After completely washing with methanol to remove unreacted 1,3-propane sultone, the solid was dried in an oven for 4 h to give SCS as a white powder.

2.4. Characterization

Fourier transform infrared spectroscopy (FT-IR) spectra were recorded on a Nicolet Nexus 870 spectrometer with KBr pellets. ^{1}H and ^{13}C NMR spectra were recorded at 343 K on a Bruker AVANCE DRX 500 (Bruker Bios pin, Rheinstetten, Germany). Molecular weight distributions were measured by a gel permeation chromatography (GPC) system equipped with a LC-20AD HPLC pump, a RID-20A RI detector, a Shodex SUGAR KS-804 linear chromatographic column in a CTO-20A thermostatic oven. GPC measurements were carried out at 60 °C using an ultrapure water solution as the eluent with a flow rate of 1.0 mL/min. The system was calibrated with Pullulan standards. Elemental analysis was conducted using an Element Vario MICRO cube elemental analyzer (Elementar, Germany), with a carrier gas (He, at a flow rate of 100 mL min^{-1}) at a combustion temperature of 1000 °C using the solid samples. The degree of sulfonation (DS) was determined by the N/S ratio based on the result of elemental analysis. DS was calculated according to Eq. (1) described by Rwei and Lien (Rwei & Lien, 2014).

$$N/S = 14 \times (0.09 \times 1 + (0.91 - X) \times 1 + X \times 1)/32 \times X, \quad DS(\%) = X \times 100\% \tag{1}$$

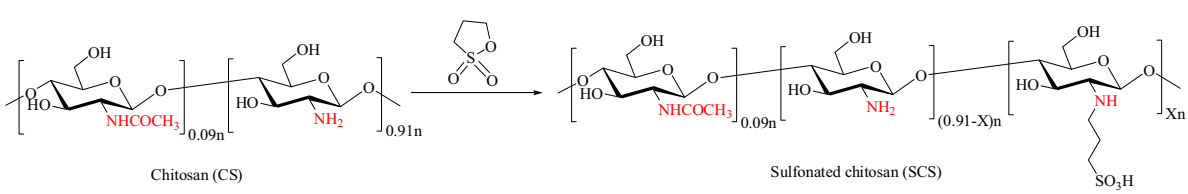
Where 14 g/mol is the molar mass of N and 32 g/mol for S, the deacetylation of chitosan is 91%.

The thermo gravimetric analysis (TGA) was performed using a USA TA thermal analyzer Model Q50, at a heating rate of 10 °C·min^{-1}, from room temperature up to 600 °C under an argon atmosphere.

2.5. Antibacterial activities of WCS and SCS against E. coli and S. aureus

2.5.1. Minimal inhibitory concentrations (MIC)

The MIC was determined according to a modified turbidimetric method in previous report (Geng et al., 2013). Polymers were dissolved in sterile nutrient broth, respectively, to prepare different concentration solutions. After autoclaving at 121 °C for 15 min, samples were inoculated with 100 μL of the freshly prepared bacteria suspension (1×10^{6} CFU/mL) in 96 well-microtitre plates and incubated at 37 °C for 24 h. The growth of bacteria was determined by optical density, as measured at 600 nm using a Microplate reader



Scheme 1. Synthesis of sulfonated chitosan (SCS).

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