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Preparation, characterization and antibacterial activity of water-soluble *O*-fumaryl-chitosan

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ABSTRACT

A novel water-soluble chitosan derivative, *O*-fumaryl-chitosan (OFCS), was prepared by using the selective partial acylation of chitosan and fumaric acid in the presence of H_2SO_4. The chemical structure and physical properties of OFCS were characterized by FTIR, ^{1}H NMR, ^{13}C NMR, and TG techniques. Our results showed that the degree of substitution (DS) for the chitosan derivatives was from 0.07 to 0.48 and the prepared derivatives had good solubility over a wide pH range, for example, the solubility of OFCS increased from 10.5 to 52.6 mg/ml as the DS increased. The antibacterial activity of chitosan and OFCS was investigated against *Escherichia coli* and *Staphylococcus aureus*. The results indicated that the antibacterial activity of OFCS was much stronger than that of chitosan and it increased with increasing DS increasing. These findings suggest that the OFCS with a DS of 0.48 is preferable for use as a food preservative.

1. Introduction

Chitosan has attracted considerable interest because of its unique combination of properties, such as biocompatibility (Rinaudo, 2006), biodegradability (Pamela et al., 2002), and antibacterial activity (Rabea, Badawy, Stevens, Smagghe, & Steurbaut, 2003). Therefore, chitosan has a variety of current and potential applications in various fields, for example, biotechnology (Mao et al., 2001), pharmaceutics (Ilium, 1998), waste water treatment (Ramnani & Sabharwal, 2006), cosmetics (Kumar, Muzzarelli, Muzzarelli, Sashiwa, & Domb, 2004), and food science (Chien, Sheu, & Yang, 2007). The antimicrobial activity of chitosan against a variety of bacteria and fungi is well known for its poly-cationic nature (Fujimoto, Tsuchiya, Terao, Nakamura, & Yamamoto, 2006; Hayashi, Ohara, Ganno, Ishizaki, & Yanagiguchi, 2007; Li, Wang, Chen, Huangfu, & Xie, 2008; Liu et al., 2006; Xu, Zhao, Han, & Du, 2007). However, this activity is limited to acidic conditions due to its poor solubility above pH 6.5. Recent studies have focused on the preparation of chitosan derivatives soluble in water, such as hydroxyethylacryl-chitosan (Ma et al., 2008), ethylamine-hydroxyethyl chitosan (Xie, Liu, & Chen, 2007), carboxymethyl-chitosan (Anitha et al., 2009), and hydroxypropyl-chitosan (Peng, Han, Liu, & Xu, 2005). However, the antibacterial activities of these water-soluble derivatives are still low. Therefore, synthesizing a new kind of derivatives that contain both antibacterial and water-soluble groups may be helpful for using chitosan as a preservative.

Fumaric acid is a food-grade acidulant with strong bactericidal activity because of its double bond and two carboxylic groups (Chikthimmah, Laborde, & Beelman, 2003). It has been used as an antimicrobial agent against pathogenic bacteria in fresh-cut lettuce and apple cider. Treatment with 50 mM of fumaric acid for 10 min caused a 2-log decrease in populations of *Escherichia coli O157:H7* and *Salmonella typhimurium* attached to fresh-cut lettuce (Kondo, Murata, & Isshiki, 2006). In addition, its esters also have strong antimicrobial activity against food pathogenic microorganism (Wang, Sun, & Kuang, 2001).

Herein we report the preparation of a water-soluble derivative of chitosan by acylation with fumaric acid. The chemical structure and physical properties of the derivative were characterized by FTIR, ^{1}H NMR, ^{13}C NMR, and TG techniques. The solubility in water as well as the antibacterial activity against *E. coli* and *Staphylococcus aureus* was also studied.

2. Experimental

2.1. Materials

Chitosan of low molecular weight from crab shells was purchased from Nantong Shuanglin Biochemical Co. Ltd. (Jiangsu, China) and used as received. According to the company analysis, its molecular weight was 40 kDa and its degree of deacetylation was about 95%. All commercially available solvents and reagents were used without further purification.

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2.2. Synthesis

The chitosan derivative was synthesized as follows (Scheme 1): 1.7 g chitosan (10 mmol calculated as glucosamine units) was suspended in 100 ml of distilled water. Fumaric acid (1–5 equiv/glucosamine unit of chitosan) was added to this solution. Then, 5 ml of H_2SO_4 was dropwise added at room temperature. The mixture was stirred at 80 °C for 4 h and cooled to room temperature (Badawy et al., 2005). The pH was adjusted to 7.0 with $NaHCO_3$. The desired compound was precipitated in acetone, filtered and washed with ethanol to remove the unreacted acid. The precipitants were soxhlet-extracted with ethanol for 2 d and oven-dried overnight at 60 °C to obtain the product.

2.3. Characterization

2.3.1. FTIR spectroscopy

Fourier transform infrared (FTIR) spectrum was recorded on a Nicolet NexuS470 instrument (Nicolet Instrument, Thermo Company, Madison, USA). Samples were prepared as KBr pellet and scanned against a blank KBr pellet background at wave number range 4000–400 cm^{-1} with resolution of 4.0 cm^{-1}.

2.3.2. NMR spectroscopy

^1H NMR and ^{13}C NMR spectra were carried out on a Bruker AV400 MHz (Bruker, Rneinstetten, Germany). Samples were dissolved in D_2O with tetramethylsilane (TMS) as an internal standard.

2.3.3. TG analysis

Thermogravimetric (TG) analysis was performed on a Mettler Toledo TGA/SDTA851 Thermo gravimeter (Mettler Toledo Corp., Zurich, Switzerland) with STARe software (version 9.01) was used to analyze the thermal stability of the samples. Samples were heated from 50 to 500 °C at a heating rate of 10 °C/min under N_2 at 20 ml/min during the analysis.

2.4. Solubility test

One gram chitosan or derivative samples with different DS was dispersed in 10 ml of distilled water (pH 7.0) and stirred at 90 °C for 12 h. The insoluble portion was separated, washed with ethanol, and then oven-dried in vacuum. The water solubility (WS) was calculated according to the Eq. (1) (Chung & Chen, 2008). Experiments were carried out in triplicate. To evaluate the effect of pH on water solubility of derivatives, 5 g sample was dissolved in 1% HAc (100 ml). With stepwise addition of NaOH solution (1 M), the transmittance of the solution was recorded with a UV spectrophotometer at 600 nm (Chung, Tsai, & Li, 2006).

$$\text{WS}\ \ (\text{mg/ml}) = \left[\frac{1 - W}{10}\right] \times 1000 \qquad (1)$$

where W is the weight of undissolved samples (g).

2.5. Antibacterial assays

E. coli and *S. aureus* were used as the test organisms. A representative microbe colony was picked off with a wire loop, placed in nutrient broth, and then incubated in an incubator shaker at 37 °C for 24 h. By diluting with sterile normal saline (0.9%) solution, the cultures of *E. coli* and *S. aureus* containing 10^7 CFU/ml were prepared and used for the antibacterial test.

The bacterial suspension (0.1 ml) was inoculated under aseptic condition into 10 ml liquid peptone medium (1% peptone, 0.3% beef extract, and 0.5% NaCl) containing chitosan or OFCS that had been adjusted to pH 7.0 and sterilized at 121 °C for 20 min. Each sample contained test materials at a concentration of 10 mg/ml, whereas, the control contained only nutrient broth without test materials. Samples were incubated shaking at 37 °C during which, a 0.1 ml of each suspension was taken to determine the bacteria count by serial dilution with triplicate plating on agar plates. After incubation for 24 h at 37 °C, the surviving colonies were counted and the mean number of the cells was calculated. The inhibition rate (IR) of the samples for different incubation times was using modified Eq. (2) according to Chung and Chen (2008).

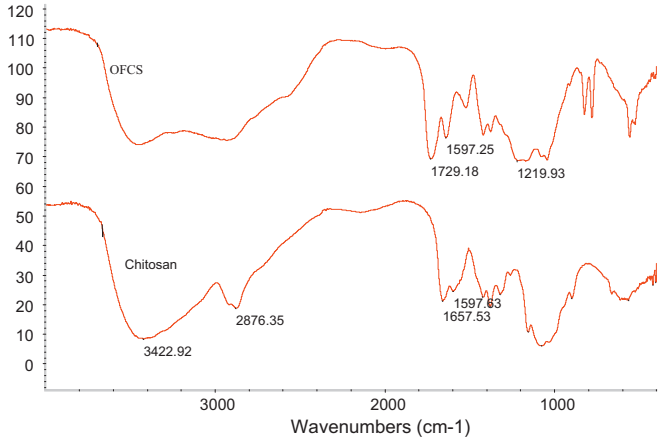


Fig. 1. FTIR spectrum of OFCS and Chitosan.

$$\text{IR}\ \ (\%) = \left[\frac{N_1 - N_2}{N_1}\right] \times 100\% \qquad (2)$$

where N_1 is the initial cell number (CFU/ml) and N_2 is the cell number after treatment (CFU/ml).

3. Results and discussion

3.1. Characterization

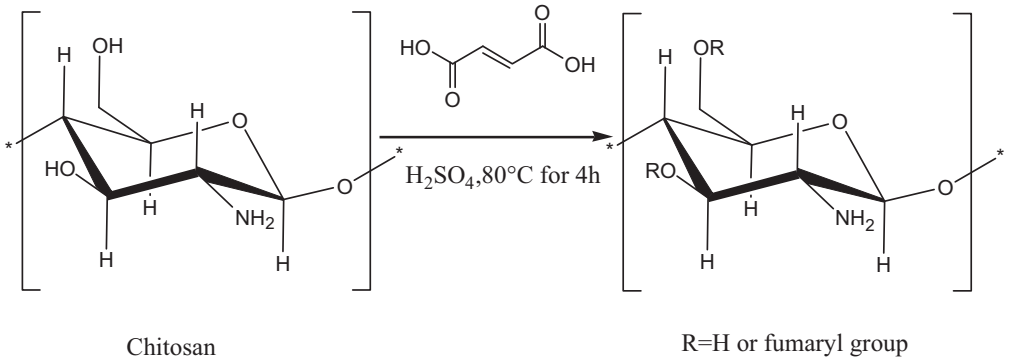
3.1.1. FTIR analysis

FTIR spectra of OFCS and chitosan are shown in Fig. 1. From the FTIR spectrum of chitosan, the broad band at around 3422 cm^{-1} was attributed to –NH and –OH stretching vibration, as well as inter- and extra-molecular hydrogen bonding of chitosan molecules. The characteristic bands at 1657, 1597, and 1320 cm^{-1} were attributed to the amide one, the amine $-NH_2$ and amide three absorption of chitosan, respectively (Zhang, Ping, Zhang, & Shen, 2003). From the FTIR spectrum of OFCS, the new absorption band at 1722 cm^{-1} was attributed to the characteristic band of ester bond, which was brought by the acylation reaction of chitosan and fumaric acid. The characteristic absorption band of NH_2 at 1597 cm^{-1} was still found. These results indicated that the acylation mainly occurred on the hydroxyl group of chitosan.

3.1.2. NMR analysis

The ^{13}C NMR spectrum of OFCS is shown in Fig. 2. The Chemical shifts δ = 97.2, 76.3, 72.5, 69.7, 62.9, and 55.6 ppm were assigned to C1, C4, C5, C3, C6, and C2 of the chitosan molecule. The chemical shifts δ = 169.2 and 166.4 ppm were assigned to carbonyl carbon atom =CH–COOH and =CH–COOR of fumaryl group. The chemical shifts δ = 134.7 and 131.4 ppm were respectively corresponding to alkenyl carbon atom of =HC–COOR and =HC–COOH of fumaryl group. The chemical shifts δ = 22.1 and 170.8 ppm indicated the existence of acetyl groups.

The data of ^1H NMR of OFCS are that δ = 2.08 ppm (s, NHAc), δ = 3.26 ppm (br s, H-2 of GlcN residue), 3.57–4.92 ppm (m, H-2 of N-acylated GlcN and H-1, 3, 4, 5, 6 of GlcN unit), δ = 6.36 ppm (br s, =CH-COOR), and δ = 6.66 ppm (br s, =CH-COOH). DS of



Scheme 1. Synthesis of OFCS.

Table 1
DS and solubility of OFCS and chitosan.

Molar ratio (fumaric acid /chitosan)	DS	WS (mg/ml)
Chitosan	0	0
1:1	0.07	10.5
3:1	0.21	21.7
5:1	0.48	52.6

each functional group was estimated by ^{1}H NMR spectrum. DS (NH_2, x) was estimated from δ 3.26 (x) vs 3.57–4.92 ($6-x$). DS (NHAc, y) was estimated from δ 2.08 ($3y$) vs 3.57–4.92 (6H). DS [(NHCOR + OCOR), z] was estimated from δ 6.36–6.66 ($2z$) vs 3.57–4.92 (6H). DS (NHCOR) = $1-x-y$; DS (OCOR) = z–DS (NHCOR). The results obtained from the NMR analysis indicated that the fumaryl group was introduced into the backbone of chitosan by the acylation and the reaction mainly occurred on the hydroxyl group rather than the amino group. Although the selective *O*-acylation of chitosan in presence of H_2SO_4 (owing to the salt formation of the primary amino group with H_2SO_4) was reported previously (Badawy et al., 2005), the detailed chemical structure and the protecting effect of H_2SO_4 on the amino group was not yet clear. As listed in Table 1, OFCS with different DS were synthesized by adjusting the molar ratio of fumaric acid to chitosan. The DS of derivatives increased with increasing of molar ratio of fumaric acid to chitosan. Nevertheless, only moderate substitution of fumaryl group was obtained in derivatives even by using an excess amount (5 equiv.) of fumaric acid. In addition, two signals (δ = 6.36, δ = 6.66 ppm) with equivalent peak area were found in the ^{1}H NMR data of OFCS, which were related to =C<u>H</u>–COOR and =C<u>H</u>–COOH of fumaryl group, respectively. In the case of reactant, fumaric acid (COOH–C<u>H</u>=C<u>H</u>–COOH), the chemical shifts of these protons were identical (δ = 6.66) due to their same chemical environment. It was indicated that the peaks of these protons were separated in different chemical environment after acylation with fumaric and chitosan. These results suggested that fumaric acid had two carboxyl groups, in which one reacted with hydroxyl group of chitosan to form ester linkage and the other was not involved.

3.1.3. TG analysis

Thermographs of (a) OFCS and (b) chitosan are shown in Fig. 3. In the case of chitosan, an estimated weight loss of about 6.8% at 90 °C was observed corresponding to the water content in the chitosan sample. A further maximum weight loss of 59% at about 260 °C was observed as a result of decomposition. It had a rapid decomposition between 250 and 350 °C, reaching its peak temperature at 310 °C. OFCS was also had two degradation stages, weight loss of

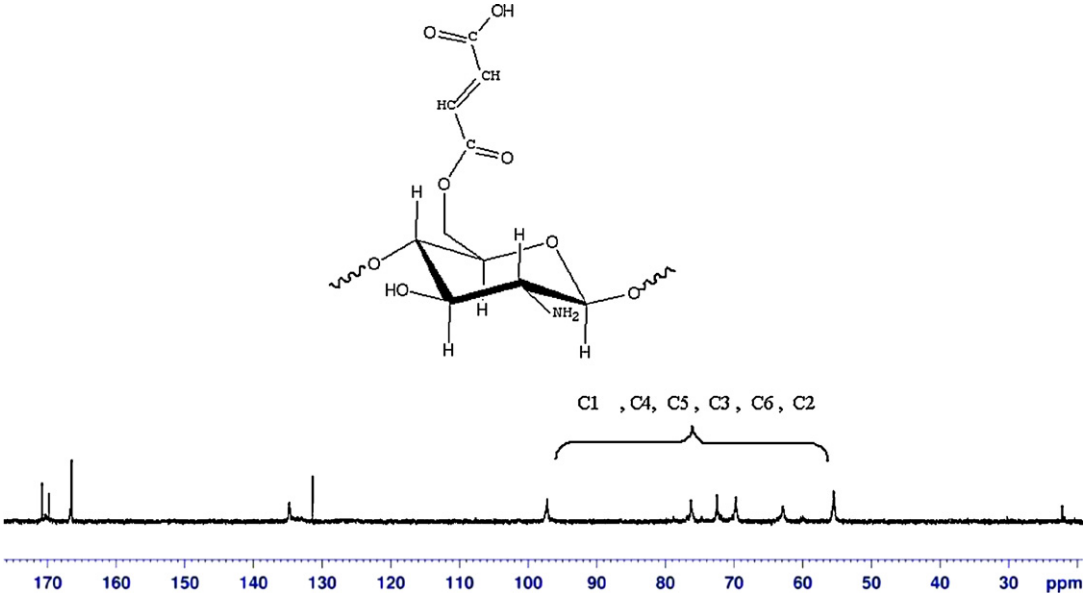


Fig. 2. ^{13}C NMR spectrum of OFCS.

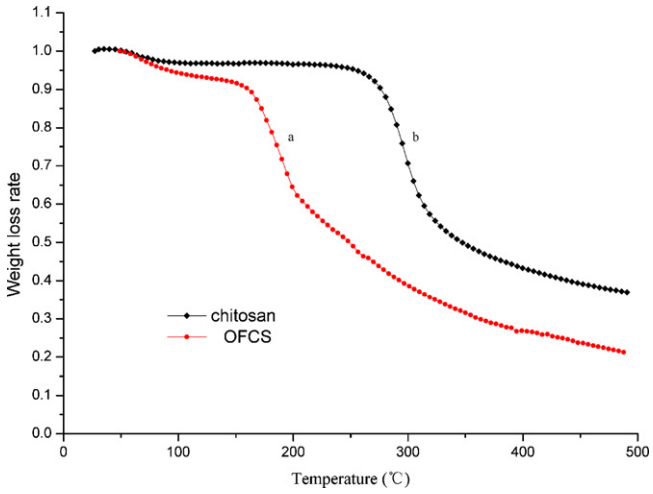


Fig. 3. Thermo gravimetric curves of (a) OFCS and (b) Chitosan.

6.8% at about 82 °C was ascribed to the volatile low molecular products and water, and weight loss of 71% from 160 °C to 250 °C which could be attributed to a complex process including dehydration of the saccharide rings, depolymerization, and decomposition of the acetylated units of the polymer (Peniche, Zaldívar, Bulay, & Román, 1993). The results indicated that the thermal stability of OFCS was lower than that of chitosan.

3.2. Solubility test

As shown in Table 1, chitosan was insoluble in water; while the solubility of the derivatives was measurable and directly related to the degree of substitution. The solubility of OFCS increased from 10.5 to 52.6 mg/ml as the DS increased. It could be elucidated that the introduction of fumaric acid with a good hydration capacity greatly decreased the intermolecular and intramolecular hydrogen bonds of chitosan. The effect of pH on water solubility of chitosan and OFCS with different DS is shown in Fig. 4. At low pH (pH < 6.0), the transmittance for the chitosan solution as well as the OFCS solutions was close to 100%. This indicated that the chitosan and OFCS molecules had good solubility in acidic conditions. While the transmittance of the chitosan solution rapidly decreased and the solution became opaque as the pH was increased from 7.0 to 9.0. In contrast, the transmittance of OFCS solutions slowly decreased, with a smaller decrease when the DS of OFCS increased from 0.07 to 0.48. These results suggested that OFCS had a better solubility than chitosan in basic conditions.

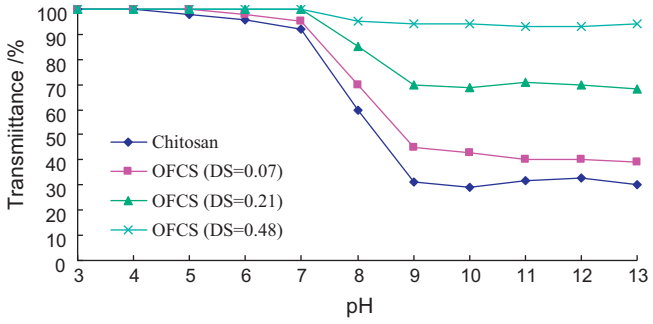


Fig. 4. pH dependence of water solubility of OFCS and Chitosan.

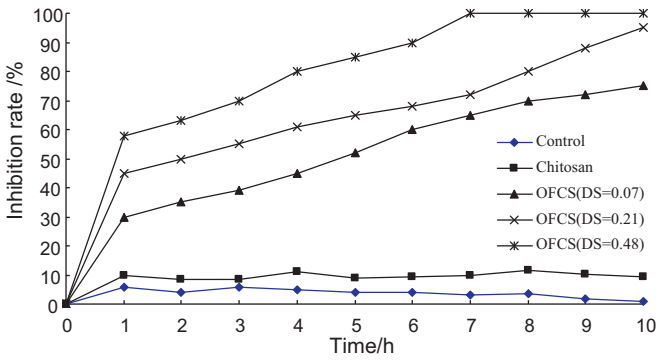


Fig. 5. Antibacterial activity of OFCS and chitosan against *E. coli*.

3.3. Antibacterial assays

The antibacterial activities of OFCS and chitosan against *E. coli* and *S. aureus* are shown in Figs. 5 and 6, respectively. It was found that chitosan gave a less inhibitory effect on the tested organism. This result accorded with the previous studies (Vishu Kumar, Varadaraj, Gowda, & Tharanathan, 2007; Xing et al., 2009) and it could be explained in that the antibacterial activity of chitosan depended on the protonation of amimo group that could interact with the negatively charged cell surface and lead to damage of the microbial cell. At pH 7.0, chitosan was not antibacterially active since it was not soluble and therefore the amino group was not positively charged. It was observed that OFCS was completely soluble at pH 7.0 and was very effective in decreasing the viable cell population for both of *E. coli* and *S. aureus*. It was also shown that the increase in DS caused higher inhibition of viable cell population of both test organisms. OFCS (DS = 0.07) caused only an inhibition of 30% in the viable cell population of the test organisms within 1 h and was unable to completely kill them during the incubation. In the case of OFCS (DS = 0.48), a decline of nearly 60% in the viable cell population of the test organism was obtained within 1 h and no viable cell population in the test solution was observed after 7 h. The antibacterial mechanism of OFCS was well interpreted by the fact that the degree of protonation of NH_2 is very low at pH 7.0, and thus the repulsion involving NH_3^+ is weak, and the strong intermolecular and intramolecular hydrogen bond results in the formation of a hydrophobic micro-area in the polymer chain. Furthermore, the carboxyl group in the polymer chain is strongly hydrophilic. Therefore, the polymer chains have hydrophobic and hydrophilic parts that provide structure affinity between the cell walls of the bacteria and the chitosan derivative (Sun, Du, Fan, Chen, & Yang, 2006; Xie, Xu, Wang, & Liu, 2002).

Several researchers have developed methods to improve the antimicrobial activity and water solubility of chitosan using chemical modifications. Chitosan oligomers have good water-solubility

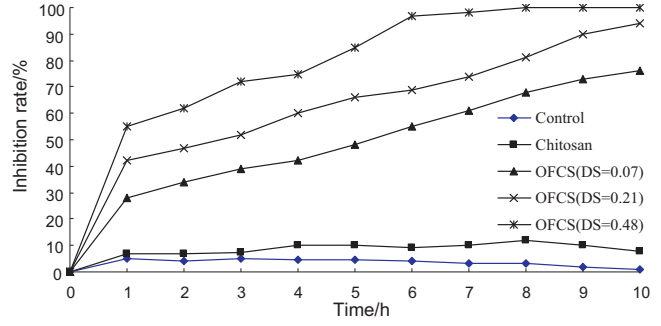


Fig. 6. Antibacterial activity of OFCS and chitosan against *S. aureus*.

because of their lower Mws. Mild hydrolysis of chitosan increased its inhibitory activity against some species of spoilage yeasts grown in complex media, whereas highly degraded chitosan oligomers showed no antimicrobial activity (Knill et al., 2004). Daly and Manuszak-Guerrini (2001) reported MIC values as low as 10–20 μg/ml against *E. coli*, *S. aureus* and *Pseudomonas aeruginosa* for N-[(2-hydroxy-3-trimethylammonium) propyl] chitosan chloride at pH 7.2. Guanidinylated chitosans were prepared and had antibacterial activity at a wide range of pH, and and they inhibited the growth of two Gram-positive bacteria *S. aureus* and *B. subtilis* at pH 6.6 (Hu et al., 2007). Qin et al. (2004) prepared a water-soluble chitosan by reaction of chitosan with glycidyl trimethylammonium chloride and had better antimicrobial activity against microorganisms under weak basic conditions compared with weak acid conditions. However, these derivatives were generally not preferred for food applications because of the formation of potential detrimental products.

4. Conclusion

O-Fumaryl chitosan (OFCS) derivatives with different DS from 0.07 to 0.48 were prepared by using acylation of chitosan and fumaric acid in the presence of H_2SO_4. FTIR, ^1H NMR, and ^{13}C NMR spectra confirmed that the fumaryl group was selectively acylated onto the hydroxyl group of chitosan. TG analysis showed that the thermal stability of OFCS was lower than that of chitosan. OFCS had good solubility in a wide pH range, which was related to the degree of substitution. OFCS exhibited higher inhibition effect than chitosan against *E. coli* and *S. aureus*, the antibacterial activity increased with the increase of DS. These results suggested that OFCS has the potential of becoming alternatives for food preservation instead of some harmful bactericides for its higher antibacterial activity.

Acknowledgements

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