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Chitosan grafted monomethyl fumaric acid as a potential food preservative

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ABSTRACT

The present study aims at *in vitro* antibacterial and antioxidant activity evaluation of chitosan modified with monomethyl fumaric acid (MFA) using 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDC) as mediator. Three different kinds of chitosan derivatives Ch-Ds-1,Ch-Ds-2 and Ch-Ds-3 were synthesized by feeding different concentration of MFA. The chemical structures of resulting materials were characterized by ^{1}H NMR, ^{13}C NMR, HR-XRD, FT-IR and TNBS assay. The results showed that Ch-Ds-1, Ch-Ds-2 and Ch-Ds-3 were successfully synthesized. The % amino groups of chitosan modified by MFA were evaluated by TNBS assay and ranging from $1.82 \pm 0.05\%$ to $7.88 \pm 0.04\%$. All the chitosan derivatives are readily soluble in water and swelled by dimethyl sulfoxide (DMSO), toluene and dimethyl formamide (DMF). The antioxidant activity for all the chitosan derivatives have been significantly improved ($P < 0.05$) compared to the chitosan. Upon antibacterial activity at pH 4.0, all the chitosan derivatives showed significant ($P < 0.05$) antibacterial activity against Gram positive *Staphylococcus aureus*, *Listeria monocytogenes* strains and Gram negative *Escherichia coli* and *Salmonella enteritidis* strains compared to chitosan. In conclusion, MFA modified chitosan has shown enhanced activities along with solubility, and could be used as a novel food preservative and packaging material for long time food safety and security.

1. Introduction

Foodborne diseases are a serious and continuously growing problem around the world. Foodborne diseases cause an estimated 48 million illness, along with 0.128 million hospitalization, and 3000 death annually in the United States alone (CDC, 2015). It is estimated that more than 250 known diseases are transmitted *via* contaminated food (OSHA, 2015). For this purpose, many food preservative have been applied in food industry to kill or inhibit microbial growth in and on foods (Sofos, 2008). Therefore, preservative agents determining food safety and shelf life of food products must meet the safety requirements. Chitosan is a natural antimicrobial, obtained from deacetylation of chitin and can be applied to extend the shelf life of different categories of food materials. The antioxidant, antimicrobial and antifungal activities of chitosan have been studied by several researchers, with particular emphasis on their ability as a food preservative (Hafdani & Sadeghinia, 2011; López-Mata et al., 2015; Srinivasa & Tharanathan, 2007; Yuan, Lv, Yang, Chen, & Sun, 2015). Being a biodegradable, edible, non-toxic, and biocompatible (Khan & Oh, 2016; Khan, Khan, Umar, & Oh, 2015; Satheeshababu & Shivakumar, 2013), chitosan has received a lot of attention for various application in food industry, pharmaceutical, optical, agriculture, biomedical, and environmental protection fields for past two decades (Hu, Wang, Li, Zeng, & Huang, 2011; Li & Huang, 2012; López-Mata et al., 2015; Szymańska & Winnicka, 2015; Yong, Shrivastava, Srivastava, Kunhikrishnan, & Bolan, 2015). However, the insolubility of chitosan in aqueous solutions (pH > 6.0) reduces some of these potential uses. On increasing pH, chitosan precipitates from acidic aqueous solutions (Bulut & Karaer, 2015; Sogias, Khutoryanskiy, & Williams, 2010). To overcome this problem, several approaches were applied to enhance the solubility of chitosan through attachment of polar functional groups to the chitosan backbone chain. Chitosan bears two types of reactive functional groups, two hydroxyls at the 3,6-carbon position and an aminogroup at the 2-carbon position which is considered to be necessary for biological activities such asantibacterial, antifungal or antiviral activity (Kurita, Ikeda, Yoshida, Shimojoh, & Harata, 2002). Modification of chitosan allows the production of

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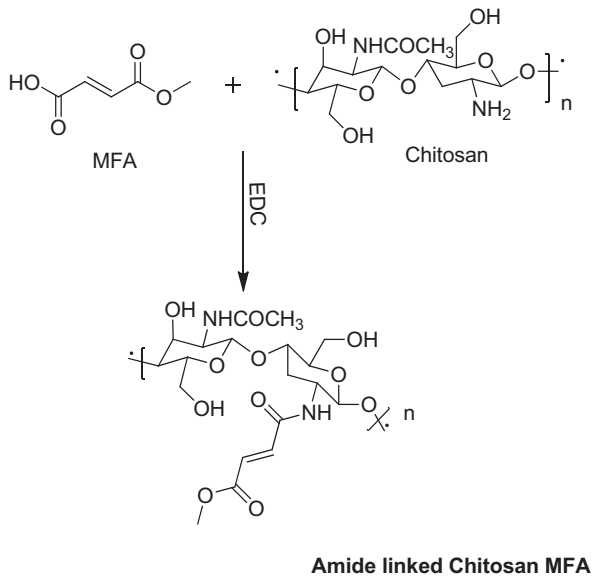

Fig. 1. Schematic representation of chitosan-MFA synthesis.

functional derivatives bycovalent binding of a molecule onto the chitosan backbone. Recently researchers have also shown that chitosan modification with desired functional group would improve water solubility and bioactivities such as antioxidants and antibacterial properties (Feng and Xia, 2011; Wang et al., 2015).

Previously, chitosan was modified with antibacterial fumaric acid (Feng & Xia, 2011) and monomethyl fumaric acid (MFA) (Wang et al., 2015), while using H_2SO_4 and 1-sulfobutyl-3-methylimidazolium trifluoromethanesulfonate ([BSmim] CF_3SO_3) respectively as reaction medium. Both the solubility and antibacterial activity of the chitosan have been effectively improved. MFA is a powerful antioxidant and good antibacterial agent. MFA has shown no toxicity and can be used as food preservative.

So far, there has been scarce report on studies using diluted acidic aqueous solution as areaction system for preparing chitosan-MFA derivative. In this work, chitosan was modified with MFA in aqueous solution and the synthesis was mediated by EDC. The modification reaction between chitosan and MFA was controlled by TNBS assay.

The chemical structures of the chitosan and Ch-Ds-2 were subsequently characterized using nuclear magnetic resonance spectroscopy (NMR), X-ray diffraction (XRD) and Fourier transform infrared spectroscopy (FT-IR). Solubility, antibacterial and antioxidant activities of the chitosan and its derivatives have been studied by solubility tests, standard plate count method, and 1,1-diphenyl-2-picrylhydrazyl (DPPH) assay respectively.

2. Materials and methods

2.1. Materials

Chitosan (low molecular weight = 52 kDa) with a degree of deacetylation (DDA) of 85%. EDC, Sodium tripolyphosphate (TPP), MFA and DPPH were purchased from Sigma-Aldrich (USA). Dialysis Tubing Cellulose (MWCO: 12–14 kDa) was purchased from Membrane Filtration Products, Inc. (USA). Picrylsulfonic acid (TNBS) solution was purchased from Tokyo Chemical Industry(Tokyo, Japan). Tryptic soy broth (TSB), tryptic soy agar (TSA) and buffered peptone water (BPW) were obtained from Difco, Sparks, MD, USA. 96 well-plates were obtained from SPL, Life Science, South Korea. Chitosan was oven dried at 60 °C for 24 h before use. All the other solvents and chemicals used in the experiments were of analytical grades and used as such without further purification.

2.2. Chitosan-MFA conjugation

Chitosan was modified with MFA according to the procedure previously described with slight modification (Wang et al., 2015). Briefly, chitosan (10 mmol as per glucosamine units) was dissolved in 100 mL aqueous solution (pH 3.0; HCl 0.1 M) and titrated with an aqueous NaOH solution (0.1 M). Three kinds of chitosan derivatives Ch-Ds-1, Ch-Ds-2 and Ch-Ds-3 were synthesized using MFA and EDC at ratio 1:1, 1:3 and 1:5 to chitosan, respectively and dissolved in distilled water. MFA and EDC were kept on stirring for 20 min to activate the carboxylic group. Both chitosan and MFA/EDC solutions were mixed and pH was adjusted to 6.0. The reaction mixtures were stirred for 24 h at room temperature. To remove the unbound EDC, the solution was dialyzed using dialysis membrane against distilled water for 72 h. After every 12 h, the dialysis medium was replaced with a fresh one. After dialysis the reaction mixtures were kept at −80 °C for 24 h followed by freeze-drying (0.05 mbar at −40 °C) for 24 h.

2.3. In situ *monitoring the synthesis of chitosan derivative*

The reaction between chitosan and MFA was *in situ* monitored by TNBS assay. TNBS form a highly chromogenic derivative when reacted with primary amines. Briefly, stock solutions of sodium bicarbonate (4%, pH 8.5), TNBS (0.01%) and HCl (1 N) were prepared. A concentration of 1 mg/mL from the reaction mixture was taken using micro pipette at various time intervals and added to the sodium bicarbonate to make a total volume of 0.250 mL. Subsequently, 0.250 mL of TNBS (0.01%) was added, and incubated at 37 °C for 2 h. The reaction was terminated by addition of 0.250 mL HCl and the absorbance of the sample was measured spectrophotometrically at 344 nm using UV–vis spectrophotometer (EppendorfBiospectrometer, USA).

2.4. Free amino group determination

Free amino groups of the chitosan and Ch-Ds-1, Ch-Ds-2 and Ch-Ds-3 were determined through TNBS assay according to the procedure previously described with slight modifications (Hardiansyah et al., 2015). Briefly, chitosan and Ch-Ds 1, Ch-Ds 2 and Ch-Ds 3 at 1 mg/mL were mixed with a mixture 0.25 mL of 4% $NaHCO_3$ and 0.25 mL of 0.01% TNBS. The reaction mixture was incubated at 37 °C for 2 h. The reaction was terminated by adding 0.250 mL of 1 N HCl. The absorbance was determined at 344 nm using UV–vis spectrophotometer (Eppendorf Biospectrometer, USA). The % amino groups of chitosan modified by MFA as a percentage were determined as follows:

$$\%substitution = \left(\frac{A-B}{A}\right) 100\%$$

Where A is the absorbance of chitosan, B is the absorbance of Ch-Ds 1, Ch-Ds 2 and Ch-Ds 3.

2.5. Antioxidant activity

The free radical scavenging activity of chitosan and Ch-Ds-1,Ch-Ds-2 and Ch-Ds-3 were determined according to the procedure previously described with slight modifications (Wang et al., 2015). Briefly, stock solution of DPPH (0.002%) and test samples (5 mg/mL) were prepared in methanol and DMSO, respectively. Test samples were mixed with 1 mL of DPPH and incubated in dark for 30 min at 23 ± 2 °C. Optical densities of the samples were measured with a UV–vis spectrophotometer (Eppendorf Biospectrometer, USA) at