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Chitosan–hydroxycinnamic acid conjugates: Preparation, antioxidant and antimicrobial activity

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ABSTRACT

In this study, the antioxidant and antimicrobial activities of chitosan–caffeic acid, chitosan–ferulic acid, and chitosan–sinapic acid conjugates with different grafting ratios were investigated. The synthesized chitosan–hydroxycinnamic acid conjugates were verified by performing ^{1}H NMR and differential scanning calorimetry analysis. The antioxidant activities of the conjugates were increased compared to the unmodified chitosan, by 1.79-fold to 5.05-fold (2,2-diphenyl-1-picrylhydrazyl scavenging assay), 2.44-fold to 4.12-fold (hydrogen peroxide scavenging assay), 1.34-fold to 3.35-fold ($ABTS^{+}$ radical scavenging assay), and also exhibited an increased reducing power. The conjugates also showed excellent lipid peroxidation inhibition abilities in a linoleic acid emulsion system. The conjugates exhibited antimicrobial activity against 15 clinical isolates, two standard methicillin-resistant *Staphylococcus aureus* (MRSA) and three standard methicillin-susceptible *S. aureus* strains, as well as eight foodborne pathogens. Additionally, the conjugates showed no cytotoxic activity towards human Chang liver and mouse macrophage RAW264.7 cells.

1. Introduction

Currently, natural antioxidants are widely used as ingredients of functional foods, with the aim of preventing chronic diseases, such as cancer, atherosclerosis and heart disease (Bengmark, Mesa, & Gil, 2009; Del Rio, Costa, Lean, & Crozier, 2010; Soory, 2009). Phenolic compounds are widely accepted as naturally occurring antioxidants, which can delay or prevent oxidative damage by reactive oxygen species (ROS), and their abilities are strongly associated with hydrogen donating and/or electron donating ability to ROS. Hydroxycinnamic acids such as caffeic acid, ferulic acid and sinapic acid are naturally occurring phenolic compounds, which have a C6–C3 skeleton. They are widely distributed in the plant kingdom, and obtained from food, such as coffee, olive oil, apple, peanut and vinegar (Aytekin, Morimura, & Kida, 2011). They have been pharmacologically evaluated in the past to reveal potent antioxidant, anti-inflammatory, and neroprotective effects (Kikuzaki, Hisamoto, Hirose, Akiyama, & Taniguchi, 2002; Kim et al., 2010; Yun et al., 2008).

Microbial contamination in foods not only results in food deterioration and a reduction of shelf life, but also leads to disease and economic losses. The growth of microorganisms in food products may cause intestinal disorders, vomiting and diarrhea (Zeng et al., 2011). Moreover, the increasing antibiotic resistance of some pathogens is a major problem throughout the world (Kaplan & Mason, 1998), and the emergence of antibiotic-resistant microorganisms has decreased the treatment options. Among them, infection of methicillin-resistant *Staphylococcus aureus* (MRSA) has become a serious worldwide problem due its high mortality rates, in spite of the available effective treatments (Eom, Kim, & Kim, 2012). Thus, there has been an increased interest in the development of antimicrobial substances from natural products and/or their derivatives as possible alternatives.

Chitosan is a naturally occurring mucopolysaccharide, and has low-toxicity, as well as being biodegradable and biocompatible. Several bioactivities, such as antioxidant, anticancer, antimicrobial, and enzyme inhibition effects have also been reported, and its unique bioactivities have been leading to its applications in the pharmaceutical industry (Cho, Kim, Ahn, & Je, 2011; Lee et al., 2009; Lee, Ryu, Je, & Kim, 2011; Muzzarelli & Muzzarelli, 2005). Therefore, there is a growing interest in developing novel chitosan derivatives with new functionality. To do so, it is generally necessary to conjugate appropriate moieties onto the chitosan backbone. In the present work, the hydroxycinnamic acids including caffeic acid, ferulic acid, and sinapic acid, were conjugated onto chitosan backbone, and antioxidant, antimicrobial and cytotoxicities of chitosan–hydroxycinnamic acid (CHA) conjugates were evaluated.

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2. Materials and methods

2.1. Materials

Chitosan (average MW 310 kDa and 90% degree of deacetylation) was donated from Kitto Life Co. (Seoul, Korea). Hydroxycinnamic acids (HAs) including caffeic acid, ferulic acid, and sinapic acid, as well as 2,2-diphenyl-1-picrylhydrazyl (DPPH), 2,2-azinobis(3-ethylbenzthiazoline)-6-sulfonic acid (ABTS), and peroxidase were purchased from Sigma Chemical Co. (St. Louis, MO, USA). The other materials required for culturing cells were purchased from Gibco BRL, Life Technologies (USA). All other chemicals and reagents used in this study were of analytical grade and commercially available.

2.2. Preparation of CHA conjugates

CHA conjugates were prepared according to our previous method, with a slight modification (Cho et al., 2011). 0.25 g of chitosan was dissolved in 25 ml of 2% acetic acid, and 0.5 ml of 1.0 M hydrogen peroxide containing 0.054 g of ascorbic acid was then added. After 30 min, each HA was added to the mixture at different amounts with the following molar ratios of chitosan repeat unit to HA; 1:0.1, 1: 0.5, and 1:1. These CHA conjugates were designated as chitosan–caffeic acid (CCA) (I), chitosan–ferulic acid (CFA) (I), and chitosan–sinapic acid (CSA) (I) with 1:0.1 molar ratio, and CCA (II), CFA (II), and CSA (II) with 1:0.5 molar ratio, and CCA (III), CFA (III), and CSA (III) with 1:1 molar ratio, respectively. The mixtures were allowed to rest at room temperature for 24 h, and were then dialyzed to remove the unreacted HA. The unmodified chitosan was also prepared without the addition of HA (Scheme 1).

2.3. Characterization of CHA conjugates

To verify CHA conjugation, ^{1}H NMR analysis was conducted at 70 °C in 2% CD_3COOD/D_2O (v/v) using a JEOL JNM ECP-400 NMR spectrometer under a static magnetic field of 400 MHz.

Differential scanning calorimetry (DSC) analyses were performed using a SINCO, STA-650. In a standard procedure about 10.0 mg of CHA conjugate was placed inside a hermetic aluminum pan, and the pan was then sealed tightly with a hermetic aluminum lid. Thermal analyses were performed from 25 to 500 °C under a dry nitrogen atmosphere with a flow rate of 25 ml/min and a heating rate of 10 °C/min.

HA contents in CHA conjugates were determined by using the Folin–Ciocalteau method. A CHA conjugate, Folin–Ciocalteau reagent and distilled water were mixed, and then 20% sodium carbonate was added. The mixture was allowed to stand for 2 h at room temperature, and the absorbance was then measured at 720 nm using a microplate reader (SpectraMax® M2/M2^e, CA, USA). A standard curve using each HA was used for calculation of the HA content in chitosan–HA conjugates. The conjugating percentage was calculated by the following equation.

$$\text{Conjugation efficiency}\ (\%) = [\text{HA in sample}\ (\text{mg})/\text{Used HA}\ (\text{mg})] \times 100$$

2.4. Determination of antioxidant capacities

2.4.1. DPPH scavenging assay

The DPPH assay was conducted according to the method of Blois (1958) with slight modifications. A 100 μl of CHA conjugates was mixed with 100 μl of 150 μM DPPH, and was allowed to stand at room temperature (RT) for 30 min. The absorbance of the mixture was then measured at 517 nm. The DPPH scavenging activity was calculated by the following equation.

$$\text{Scavenging activity}\ (\%) = [(A_{517}\ \text{of control} - A_{517}\ \text{of sample})/A_{517}\ \text{of control}] \times 100$$

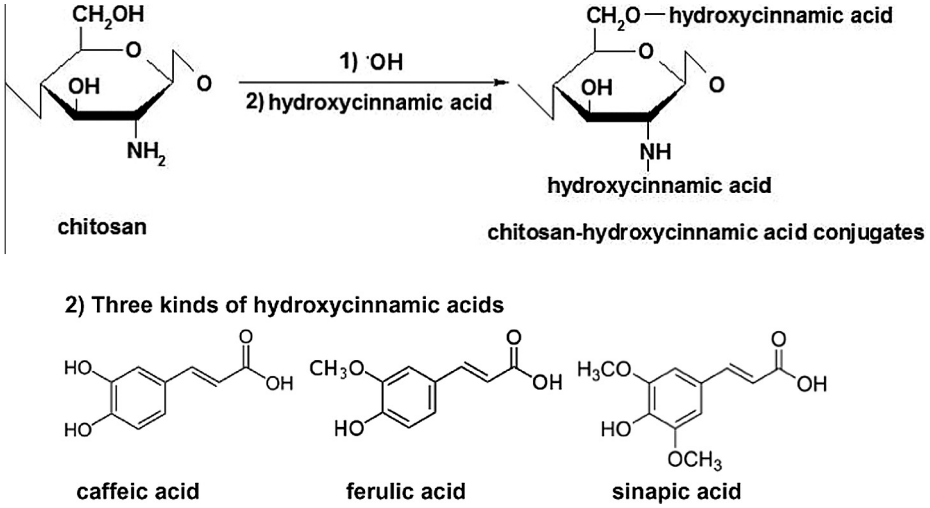
2.4.2. Hydrogen peroxide scavenging activity

The hydrogen peroxide scavenging assay was performed using the method of Müller (1985). A 100 μl of sodium phosphate buffer (0.1 M, pH 5.0), CHA conjugates, and 20 μl of hydrogen peroxide was mixed and incubated at 37 °C for 5 min. Following incubation, 30 μl of 1.25 mM ABTS and 30 μl of 1 U/ml of peroxidase were added to the mixture, which was subsequently incubated at 37 °C for 10 min. The absorbance was then measured at 405 nm using a microplate reader. The hydrogen peroxide scavenging activity was calculated by the following equation.

$$\text{Scavenging activity}\ (\%) = [(A_{405}\ \text{of control} - A_{405}\ \text{of sample})/A_{405}\ \text{of control}] \times 100$$

2.4.3. ABTS$^+$ radical-scavenging activity

The ABTS$^+$ radical-scavenging activity of CHA conjugates was evaluated according to the method of Park and Kim (2009). A stock solution of ABTS$^+$ radicals was prepared by 7 mM ABTS containing



Scheme 1. Synthesis pathway of chitosan–hydroxycinnamic acid conjugates.