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Antioxidant and Antibacterial Activities of Chitosan-Phloroglucinol Conjugate

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Abstract

In an effort to develop biopolymer-based antioxidant and antibacterial materials, a chitosan-phloroglucinol conjugate was prepared and cellular antioxidant activity and minimum inhibitory concentration against foodborne pathogens and methicillin-resistant *Staphylococcus aureus* (MRSA) evaluated. The chitosan-phloroglucinol conjugate showed higher antioxidant activities than the unmodified chitosan ($P < 0.05$). The chitosan-phloroglucinol conjugate showed 62.29% reactive oxygen species scavenging activity, 56.11% lipid peroxidation inhibition activity, and 2.21-fold increase of glutathione expression in mouse macrophage cells. Additionally, the chitosan-phloroglucinol conjugate exhibited higher antibacterial activities than the unmodified chitosan, and the chitosan-phloroglucinol conjugate showed fourfold higher antibacterial activities against MRSA and clinical isolates and twofold higher activities against foodborne pathogens compared to the unmodified chitosan.

Key words: Chitosan, Phloroglucinol, Antioxidant activity, Antibacterial activity

Introduction

Chitosan is a naturally occurring mucopolysaccharide and the second most abundant biopolymer, exhibiting versatile biological properties including biodegradability, biocompatibility, and a less toxic nature. These properties make chitosan attractive for a wide variety of pharmaceutical, biomedical, food industry, health and agricultural applications (Felt et al., 1998; Kim et al., 2007; Lin et al., 2009). Moreover, chitosan has been used for the development of new physiologically bioactive materials because it exhibits versatile biological properties, including antioxidant, antibacterial, anti-cancer, anti-inflammatory and immunostimulatory activities (Jeon and Kim, 2001; Park et al., 2004a; 2004b; Lee et al., 2009a, 2009b, 2011). However, its water-insolubility is a major limiting factor. Therefore, there is growing interest in developing novel chitosan derivatives with desired characteristics, including enhanced water solubility. Consequently, methods to improve not only the water solubility but also the biological activities of chitosan have been developed by using both chemical and enzymatic modifications. Typically, appropriate moieties are conjugated onto the chitosan backbone. Recently, our group developed a gallic acid-*grafted*-chitosan using a free radical redox/pair system, which showed improved antioxidant and antimicrobial activities compared with unmodified chitosan (Cho et al., 2011; Lee and Je, 2013). These results suggested that conjugation of chitosan with a functional molecule is a promising strategy for improving its biological activity.

Marine-derived bioactive molecules have been isolated and their bioavailability characterized. Phloroglucinol is a phytochemical derived from edible brown algae with antioxidant, tyrosinase-inhibiting, cytoprotective and anti-inflammatory activities (Kang et al., 2004, 2006; Heo et al., 2005; Yoon et al., 2009; Kim and Kim, 2010). Previously, we demonstrated

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successful conjugation of phloroglucinol onto the chitosan backbone, and the chitosan-phloroglucinol conjugate showed higher antioxidant activity *in vitro* than unmodified chitosan (Woo and Je, 2013). However, the cellular antioxidant capacity of the chitosan-phloroglucinol conjugate was not explored. Therefore, in the present study we investigated the cellular antioxidant capacity of the chitosan-phlorolgucinol conjugate, and determined the antimicrobial activities against methicillin-resistant *Staphylococcus aureus* (MRSA) and foodborne pathogens.

Materials and Methods

Materials

Chitosan (average MW, 310 kDa; degree of deacetylation, 90%) was donated by Kitto Life Co. (Seoul, Korea). Phloroglucinol, Folin-Ciocalteau phenol reagent, and hydrogen peroxide were purchased from Sigma Chemical Co. (St. Louis, MO, USA). Monobromobimane (mBBr), diphenyl-1-pyrenylphosphine (DPPP), and 2′,7′-dichlorofluorescin diacetate (DCFH-DA) were obtained from Molecular Probes Inc. (Eugene, OR, USA). The other materials required for cell culture were purchased from Gibco BRL, Life Technologies (Grand Island, NY, USA). All other chemicals and reagents used in this study were of analytical grade and commercially available.

Microorganisms and media

The bacterial strains tested for antibacterial activity were purchased from the Korean Collection for Type Cultures (KCTC; Daejeon, Korea) and 15 clinical isolates of MRSA were provided by Dong-A University Hospital (Busan, Korea). All strains were grown aerobically at 37°C in Mueller-Hinton broth (Difco, Detroit, MI, USA) and subsequently used for assays of antibacterial activity.

Preparation of chitosan-phloroglucinol conjugate

The chitosan-phloroglucinol conjugate was prepared according to our previous method with a slight modification (Woo and Je, 2013). Briefly, chitosan (0.25 g) was dissolved in 25 mL of 2% acetic acid, and then 0.5 mL of 1.0 M hydrogen peroxide containing 0.054 g of ascorbic acid was added. After 30 min, phloroglucinol (18.43 mg) was added to the mixture, and then allowed to rest at room temperature for 24 h. Unreacted phloroglucinol was removed by dialysis for 48 h using distilled water. Unmodified chitosan was also prepared without the addition of phloroglucinol. Molar ratios of repeating units of chitosan to phloroglucinol were 1:0.1, which was optimal.

To confirm successful synthesis, ^{1}H NMR analysis was conducted and the results were compared to the report by Woo and Je (2013). Unmodified chitosan: ^{1}H NMR (400 MHz, D_2O) δ: 4.90 (1H, H-1), 3.14 (1H, H-2), 3.41-4.30 (1H, H-3/6), 2.00 (H-Ac), 5.0 (D_2O). Chitosan-phloroglucinol conjugate: ^{1}H NMR (400 MHz, D_2O) δ: 6.30 and 7.58 (aromatic protons of phloroglucinol), 4.90 (1H, H-1), 3.25 (1H, H-2), 3.65-4.36 (1H, H-3/6), 2.12 (H-Ac), 5.0 (D_2O).

The phloroglucinol content of the chitosan-phloroglucinol conjugate determined using the Folin-Ciocalteau method was 29.28 ± 0.90 mg phloroglucinol/g chitosan-phloroglucinol conjugate.

Minimum inhibitory concentrations (MIC)

The twofold serial dilution method was used to determine the chitosan-phloroglucinol conjugate MIC against MRSA and foodborne pathogens as described by the National Committee for Clinical Laboratory Standards (2004). The MIC was defined as the lowest concentration that demonstrated no visible growth after incubation at 37°C for 24 h.

Determination of cellular antioxidant activities

Cell culture. Mouse macrophage cells (RAW264.7) were cultured in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% fetal bovine serum, 2 mM glutamine, 100 U/mL of penicillin, and 100 μg/mL streptomycin. The cells were incubated at 37°C in a humidified atmosphere.

Cytotoxicity assay. Cytotoxicity of the chitosan-phloroglucinol conjugate was estimated using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. RAW264.7 cells were grown in 96-well plates at a density of 1×10^4 cells/well. After 24 h, the cells were treated with the desired concentrations of chitosan-phloroglucinol conjugate and incubated at 37°C for 24 h. A 100-μL aliquot of MTT solution (1 mg/mL) was added after aspiration of medium and cells were incubated for an additional 4 h. Next, the supernatant was aspirated, and finally, a 100 μL aliquot of dimethyl sulfoxide (DMSO) was added to solubilize the formazan crystals. The quantity of formazan crystals was determined by measuring the absorbance at 540 nm using a microplate reader.

Cellular reactive oxygen species (ROS) scavenging activity. Intracellular ROS formation was assessed according to a method described previously, employing the oxidation-sensitive dye DCFH-DA as the substrate (Engelmann et al., 2005). RAW264.7 cells growing in black microtiter 96-well plates were labeled with 20 μM DCFH-DA in HBSS for 20 min in the dark. The cells were then treated with the chitosan-phloroglucinol conjugate and incubated for 1 h. After washing the cells three times with phosphated buffered saline (PBS), 500 μM H_2O_2 (in HBSS) was added. The formation of 2′,7′-dichlorofluorescein (DCF), due to the oxidation of DCFH in the presence of various ROS, was read after every

30 min at an excitation wavelength of 485 nm and an emission wavelength of 528 nm (SpectraMax M2/M2^e).

Lipid peroxidation inhibition. Lipid peroxidation inhibition was assessed by measuring the intracellular lipid hydroperoxide level using the fluorescent probe, DPPP (Takahashi et al., 2001). RAW264.7 cells growing in culture dishes were washed three times with PBS and labeled with 13 μM DPPP (dissolved in DMSO) for 30 min at 37°C in the dark. The cells were then washed three times with PBS and seeded onto fluorescence microtiter 96-well plates at a density of 4 × 10^5 cells/mL using serum-free media. Following complete attachment, the cells were treated with the chitosan-phloroglucinol conjugate and incubated for 1 h. After incubation, 3 mM AAPH in PBS was added and DPPP oxide fluorescence intensity was measured after 6 h at an excitation wavelength of 361 nm and an emission wavelength of 380 nm. The fluorescence values were normalized to cell numbers using the MTT cell viability assay.

Determination of glutathione (GSH) level. Intracellular GSH level was determined using a thiol-staining reagent, mBBr, according to a previous method with slight modification (Poot et al., 1986). RAW264.7 cells were seeded at a concentration of 4.0 × 10^5 cells/mL and following confluence, were treated with the chitosan-phloroglucinol conjugate for 1 h. The cells were then labeled with 40 μM mBBr for 30 min, and then mBBr-GSH fluorescence intensity was measured at an excitation wavelength of 360 nm and an emission wavelength of 465 nm using a spectrofluorometer (Spectra Max).

Statistical analysis

All results are expressed as means ± standard deviation (SD) of three determinations. Differences between the means of each group were assessed by one-way analysis of variance (ANOVA) followed by Duncan's test using the statistical software, PASW Statistics 19.0 (SPSS Inc., Chicago, IL, USA). A value of $P < 0.05$ was taken to indicate statistical significance.

Results and Discussion

Cellular ROS scavenging activity

Cytotoxicity was first determined in RAW264.7 macrophage cells at the desired concentration (50, 100, and 200 μg/mL) by MTT assay; the results confirmed that the chitosan-phloroglucinol conjugate did not exhibit any cytotoxic effect (Fig. 1). Cellular ROS scavenging activities of the chitosan-phloroglucinol conjugate and the unmodified chitosan were first evaluated using a fluorescent probe, DCFH-DA (Fig. 2). DCFH-DA freely penetrated into the cells and was hydrolyzed by intracellular esterases to DCFH and trapped inside the cells. DCFH was further oxidized to DCF by ROS, emitting fluorescence. Pre-treatment with the unmodified chitosan and the chitosan-phloroglucinol conjugate decreased DCF fluorescence in a dose-dependent manner. At 200 μg/mL unmodified chitosan, a 26.29% ROS scavenging activity was observed, whereas the chitosan-phloroglucinol conjugate exhibited 62.29% ROS scavenging activity at the same concentration; this difference was significant ($P < 0.05$). Thus conjugation of phloroglucinol onto chitosan improved the antioxidant activity.

Excessive production of ROS may lead to a number of de-

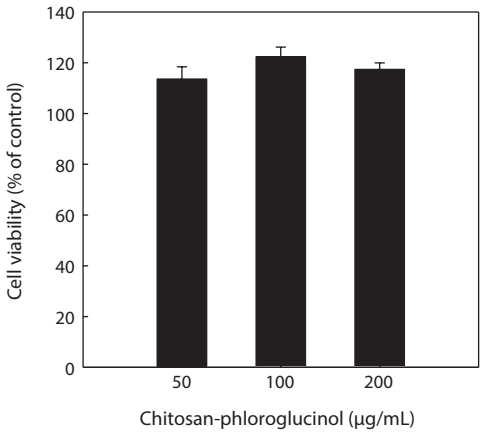

Fig. 1. Cytotoxic effect of the chitosan-phloroglucinol conjugate. All assays were done in triplicate and data are expressed as means ± SE.

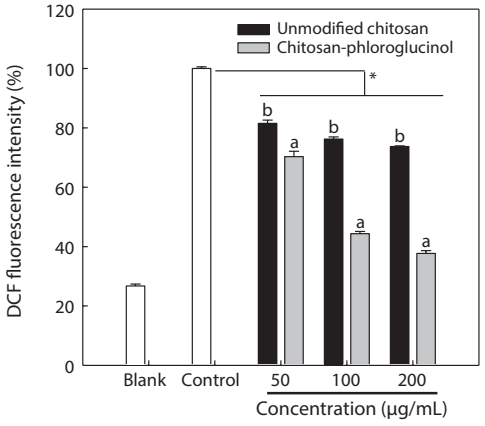

Fig. 2. Inhibition of intracellular reactive oxygen species (ROS) formation of unmodified chitosan and chitosan-phloroglucinol conjugate in mouse macrophage cells. Mouse macrophage cells were labeled with a non-toxicfluorescence dye, DCFH-DA, and treated with different concentrations of chitosan-phloroglucinol conjugate. The fluorescence intensities of DCF due to oxidation of DCFH by cellular ROS (generated by H_2O_2) were detected ($\lambda_{excitation}$ = 485 nm, $\lambda_{emission}$ = 528 nm). All assays were done in triplicate and data are expressed as means ± SE. *$P < 0.05$ vs. control. ^{a,b}The values with different subscripts indicate significant difference at the same concentration ($P < 0.05$).

generative processes, such as cancer, cardiovascular and neurodegenerative conditions, and premature aging (Halliwell and Gutteridge, 1999; Finkel and Holbrook, 2000). Additionally, a crucial step in these ROS-mediated effects is DNA damage (Halliwell and Gutteridge, 1999). Thus, prevention of ROS-induced oxidative stress may help to maintain human health, and consuming dietary antioxidants from natural sources may have health-promoting effects. In this study, we demonstrated that the chitosan-phloroglucinol conjugate effectively scavenged intracellular ROS.

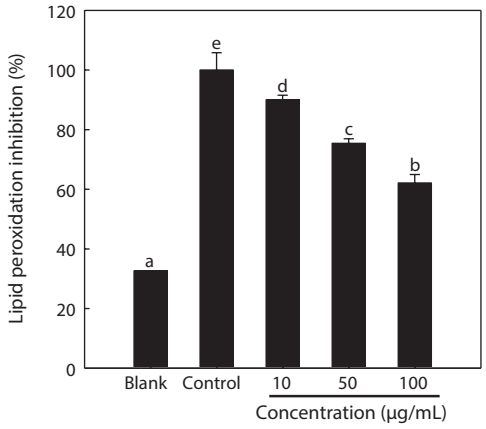

Fig. 3. Effect of chitosan-phloroglucinol conjugate on membrane lipid peroxidation inhibition as assessed by DPPP fluorescence assay. Mouse macrophage cells, treated with different concentrations of chitosan-phloroglucinol conjugate, were exposed to AAPH to initiate membrane lipid peroxidation. DPPP oxide fluorescence emitted due to the oxidation of DPPP by lipid hydroperoxides was compared with an AAPH non-treated blank group and an AAPH alone-treated control. The results are the mean ± SE of three independent experiments. $^{a\text{-}e}$The values with different subscripts indicate significant difference at $P < 0.05$.

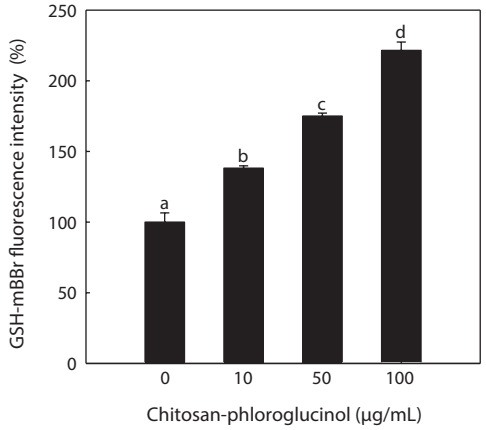

Fig. 4. Effect of chitosan-phloroglucinol conjugate on expression of glutathione (GSH) level in mouse macrophage cells.The cells were treated with predetermined concentrations of chitosan-phloroglucinol conjugate and incubated for 30 min. Cellular GSH levels weredetermined using mBBr as a thiol-staining reagent according to the method described in the text, by measuring mBBr-GSH fluorescence intensity ($\lambda_{excitation}$ = 360 nm, $\lambda_{emission}$ = 465 nm). The results are the mean ± SE of three independent experiments. $^{a\text{-}d}$The values with different subscripts indicate significant difference at $P < 0.05$.

Lipid peroxidation inhibition by the chitosan-phloroglucinol conjugate

Overproduction of ROS results in an attack of not only DNA, but also other cellular components including the polyunsaturated fatty acid residues of phospholipids, which are highly sensitive to oxidation (Siems et al., 1995). Therefore, unsaturated fatty acids in cell membranes are susceptible to free radical-mediated oxidation. The DPPP fluorescent probe was used to evaluate lipid peroxidation in the cells (Fig. 3). After exposure to AAPH, considerable lipid peroxidation was observed in the control group (3.06-fold) compared to the non-treatment group. However, pre-treatment with the chitosan-phloroglucinol conjugate significantly inhibited lipid peroxidation in a dose-dependent manner ($P < 0.05$). At 100 μg/mL, the lipid peroxidation inhibition activity of the chitosan-phloroglucinol conjugate was 56.11%.

Effect of the chitosan-phloroglucinol conjugate on GSH level

GSH is the major soluble antioxidant in cell compartments and is a key cellular reductant, reducing numerous oxidizing compounds, including ROS and lipid peroxides, and is oxidized to GSH disulfide and other mixed disulfides (Kadiska et al., 2000). The main protective roles of GSH against oxidative stress include acting as a cofactor of GSH peroxidase, which detoxifies hydrogen peroxide and lipid peroxides, directly scavenging $^{\bullet}OH$ and singlet oxygen, and regenerating vitamin C and E *via* reduction of ascorbyl and tocopherol radicals, respectively (Masella et al., 2005). GSH is produced in the liver and is present in many cells at concentrations up to 10 mM; however, many disease conditions are associated with low GSH levels, indicating oxidative stress (Reid and Jahoor, 2001). The effect of the chitosan-phloroglucinol conjugate on GSH levels in macrophage cells was determined using the thiol-reactive fluorescence dye mBBr (Fig. 4). A dose-dependent increase in GSH expression was observed in cells treated with the chitosan-phloroglucinol conjugate; at 100 μg/mL, a 2.21-fold increase in GSH occurred compared to the non-treatment group ($P < 0.05$).

Antibacterial activity of the chitosan-phloroglucinol conjugate

Chitosan exhibits antibacterial activity against a broad spectrum of foodborne pathogens; this activity is influenced by the type of chitosan, molecular weight and several other physiochemical properties (Park et al., 2004a). To date, many chitosan derivatives exhibiting antibacterial activity have

been developed by introducing specific functional groups, indicating that modification of chitosan is a good strategy for developing antibacterial biopolymers (Tang et al., 2010; Xiao et al., 2011). Additionally, we previously demonstrated that gallic acid-*grafted*-chitosan showed higher antibacterial activity than unmodified chitosan (Lee and Je, 2013). We evaluated the antibacterial activity of the chitosan-phloroglucinol conjugate against MRSA and foodborne pathogens (Table 1). First, the unmodified chitosan showed antibacterial activity against 3 methicillin-sensitive *Staphylococcus aureus* (MSSA) strains, 2 standard MRSA strains and 15 clinical MRSA isolates. The MIC values of unmodified chitosan were 64-128 μg/mL for the 3 MSSA strains, 256 μg/mL for the 2 standard MRSA strains, and 256-512 μg/mL for the 15 clinical isolates. However, the chitosan-phloroglucinol conjugate exhibited better antibacterial activity than the unmodified chitosan. The equivalent MICs for the chitosan-phloroglucinol conjugate were 32-64 μg/mL for the 3 MSSA strains, 64 μg/mL for the 2 standard MRSA strains, and 64-128 μg/mL for the 15 clinical MRSA isolates. The antibacterial activity of the chitosan-phloroglucinol conjugate was enhanced twofold for MSSA and fourfold for standard and clinical MRSA isolates compared to unmodified chitosan. Similar results were observed for foodborne pathogens (Table 1). Unmodified chitosan showed an MIC of 128 μg/mL for gram-positive bacteria (*Bacillus subtilis*, *Enterococcus faecalis*, and *Listeria monocytogenes*) and 512-1,024 μg/mL for gram-negative bacteria (*Escherichia coli coli*, *Klebsiella pneumonia*, *Legionella birminghamensis*, *Pseudomonas aeruginosa*, and *Salmonella typhimurium*), whereas the chitosan-phloroglucinol conjugate exhibited MICs of 32-64 μg/mL for gram-positive bacteria and 256-512 μg/mL for gram-negative bacteria. Therefore, the antibacterial activity of chitosan was increased by its conjugation with phloroglucinol.

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Table 1. Minimum inhibitory concentrations (MICs) of chitosan-phloroglucinol and unmodified chitosan

Strain	MIC (μg/mL)	
	Chitosan-phloroglucinol	Unmodified chitosan
MSSA (KCTC 1927)	64	128
MSSA (KCTC 1928)	64	128
MSSA (KCTC 1916)	32	64
MRSA (KCCM 40510)	64	256
MRSA (KCCM 40511)	64	256
MRSA D-1*	64	256
MRSA D-2*	128	256
MRSA D-3*	128	256
MRSA D-4*	128	256
MRSA D-5*	128	512
MRSA D-6*	64	256
MRSA D-7*	128	512
MRSA D-8*	64	256
MRSA D-11*	64	256
MRSA D-12*	128	512
MRSA D-13*	64	512
MRSA D-14*	64	512
MRSA D-17*	64	256
MRSA D-19*	64	256
MRSA D-20*	128	256
Bacillus subtilis (KCTC 1028)	64	128
Enterococcus faecalis (KCTC 2011)	32	128
Listeria monocytogenes (KCTC 3569)	64	128
Escherichia coli (KCTC 1682)	256	1024
Escherichia coli (KCTC 2571)	256	512
Klebsiella pneumonia (KCTC 2242)	256	512
Legionella birminghamensis(KCTC 2057)	512	1024
Pseudomonas aeruginosa(KCTC 1637)	256	512
Salmonella typhimurium(KCTC 1925)	256	512

MSSA, methicillin-susceptible *Staphylococcus aureus*; MRSA, methicillin-resistant *Staphylococcus aureus*.
*MRSA strains were isolated at Dong-A University Medical Hospital

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