Antioxidative Effects of Sulfated Chitooligosaccharides on Oxidative Injury

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ABSTRACT

Protective effects of sulfated chitosan and chitooligosaccharides were evaluated by employing a fluorescence activated cell sorter (FACS) on hydrogen peroxide and plasmid DNA strand breaks assay. We prepared sulfated chitosan (SC) and sulfated chitooligosaccharides (SCOS I, SCOS II and SCOS III) from 90% deacetylated chitosan and three kinds of chitooligosaccharides (COS I; 5,000-10,000 Da, COS II; 1,000-5,000 and COS III; below the 1,000). The SCOS III showed the highest protective activities in liver cells, with cells apoptosis percentage values of 22.54% at 1.0 mg/mL. In addition, the SCOS III exhibited a protective effect on H$_2$O$_2$-induced DNA damage. These results indicate that SCOS III possesses potent protective effects on liver cells and DNA against H$_2$O$_2$-induced oxidative damage.

Keywords: apoptosis, DNA protective effect, sulfated chitosan, sulfated chitooligosaccharides,

INTRODUCTION

Antioxidants are substances that delay or prevent the oxidation of cellular oxidizable substrates. They exert their abilities by scavenging free radicals and reactive oxygen species (ROS), preventing the generation of free radicals and ROS, or activating a battery of detoxifying proteins. Therefore, the role of antioxidants has received increased attention during the past decade. In general, many synthetic antioxidants such as butylated hydroxyanisole, butylated hydroxytoluene, tert-butylhydroquinone, and propyl gallate are commercially available but are quite unsafe, and their toxicity is a source of concern(1). Therefore, in recent years, considerable attention has been directed towards identification of natural products with antioxidant activity that may be used for human consumption.

Chitin, a polymer of N-acetylglucosamine (β-1,4 linked 2-acetamido-D-glucose), is a cellulose-like biopolymer present in the exoskeleton of crustaceans and in cell walls of fungi, insects and yeast. Chitosan is derived from chitin by deacetylation in the presence of alkali(2). While chitin has a limited application because of its poor solubility and reactivity, chitosan is soluble in acetic acid and other organic solvents. Therefore, chitosan has been widely used in vastly diverse fields such as pharmaceutics, medicine and biotechnology(3). Meanwhile, increasing attention has recently been given to converting its oligosaccharides because of their specific biological activities such as antitumor activity(4,5), immunostimulating effects(6,7), enhancing protective effects against infection with some pathogens in mice(8,9), antifungal activity(10,11), antimicrobial effects(12), antioxidative activity(13). However, there are little information of protective effect of chitosan and their oligosaccharide sulfates on liver cells and DNA.

In this study, the protective effects of SC and SCOSs with different chain length on the liver cells and plasmid DNA were investigated.

MATERIALS AND METHODS

Materials

Chitin prepared from crab shells was donated by Kitto Life Co. (Seoul, Korea). The chitosanase (35,000 U/g protein) derived from Bacillus sp. was purchased from Amicosen Co. (Jinju, Korea). The ultrafiltration (UF) membrane reactor system for production of COS was from Millipore Co. (Bedford, USA). All other reagents were of the highest grade available commercially.

Preparation of COSs

90% Deacetylated chitosan was prepared from crab chitin by N-deacetylation with 40% (w/v) sodium hydroxide solution for durations according to our previous method (14). In addition, the COSs were prepared by hydrolysis of chitosan in an UF membrane reactor system according to the method of Park et al. (15). One percent solution of chitosan was prepared by dispersing 100 g of chitosan in 1.0 L of distilled water, dissolving it and stirring by adding 550 mL of 1.0 M lactic acid, and making up to 10.0 L with distilled water. The pH was adjusted to be 5.5 with a saturated sodium carbonate solution. The UF membrane reactor system (Millipore Minitan, Millipore Co.) was used for preparation of COS. 90% deacetylated chitosan was hydrolyzed with an endo
type chitosanase (35,000 U/g protein) with a substrate to enzyme ratio of 1 : 1.5 units for 3 h in a batch reactor and then heated at 98°C for 10 min to inactivate the enzyme. Thereafter, the hydrolysates were separated using a UF membrane reactor system. The UF membrane used in the system were molecular weight cut-off (MWCO) 10, 5 and 1 kDa, respectively. The COSs were fractionated into three kinds of COSs with relatively high molecular weights (5,000 Da; 90-COS I), medium molecular weights (1,000-5,000 Da; 90-COS II), and low molecular weights (below 1,000 Da; 90-COS III). The molecular weights of the COSs were carried out by Park et al. (16). All COSs recovered were lyophilized on a freezing drier for 5 days.

**Synthesis of sulfated Chitosan and COSs**

Based on the method of Holme and pearls (17) with some modifications, sulfated chitosan and COSs were prepared. That is, 10 g of chitosan and COS which had previously been lyophilized, was dispersed in 1 L of distilled water, and treated with 22 g of sodium carbonate anhydrous and 45 g of trimethylamin-sulfur trioxide (Me₃N-SO₃). The mixture was heated at 65°C until a clear viscous solution or gel formed for 12 h. The cooled mixture was then dialyzed exhaustively against distilled water using an electronic dialyzer (Micro Acilyzer G3, Asahi Chemical Industry Co., Tokyo, Japan), and lyophilized. The dialyzer membrane used was Aciplex Cartridge (AC-230-400). Characteristic absorptions derived from the sulfogroups in the IR spectrum at 800, 1,240 and 1,350 cm⁻¹ were assigned to C-O-S, S = O and S-N, respectively (18). All sulfated chitosan and sulfated COSs were lyophilized on a freezing drier for 5 days.

**Cell culture**

Chang cells used in this study were obtained from the American Type Culture Collection, and were used for no more than 10-12 passages. Growth medium consisted of DMEM supplemented with 10% heat-inactivated fetal bovine serum (FBS), as well as antibiotics (10 µg/mL penicillin-streptomycin). The cells were incubated in a humidified incubator at 37°C with 5% CO₂ and 95% air. All treatments were performed at 30% confluence of cells.

**Flow cytometry**

For sub-G1 and cell cycle analysis, chang cells were suspended in ethanol with 0.5% Tween-20 and left for 24 hr at 4°C. The cells were harvested by centrifugation and resuspended in 1.0 mL of PBS with 0.05 mg/mL of propidium iodide and 10 µg/mL of RNase A, and incubated at 37°C for 30 min. The analysis of apoptotic cell death was performed by measuring the hypodiploid DNA contents using a flow cytometer (FACS-caliber, Becton Dickinson, NJ). The cells in sub-G1 population were considered as apoptotic cells and percentage of each phase of cell cycle was determined.

**Protective Effect of SCOS III on DNA Damage Induced by H₂O₂**

To study the protective effects of 90-SCOS III on DNA damage induced by H₂O₂, the reaction was conducted in an Eppendorf tube (Hamburg, Dentsand, Germany) at a total volume of 13 µL containing 0.5 µg of pBR 322 DNA in 3 µL of 50 mM phosphate buffer (pH 7.4), 3 µL of 2 mM FeSO₄ and 2 µL of the 90-SCOS III at various concentrations. Then 4 µL of 30% H₂O₂ was added, and the mixture was incubated at 37°C for 1 hr. The mixture was subjected to 0.8% agarose gel electrophoresis, and DNA bands (supercoiled [SC], linear and open circular [OC]) were stained with ethidium bromide. SC DNA was quantified by Image Guage (Fujifilm, Japan).

**Statistical analysis**

The data were presented as mean±standard deviation (SD). The paired t-test was used for comparisons between damage group and 90-SCOS III-treated group. All analyses were performed using an Self-Propelled Semi-Submersible (SPSS) system (SPSS, Chicago, IL).

**RESULTS AND DISCUSSION**

**Protective effect of SCOS III on H₂O₂-Induced damage**

Due to its strong reactivity with biomolecules, -OH is probably capable of doing more damage to biological systems than any other ROS (19). Therefore, sulfated chitosan and sulfated COSs which have the highest alkyl radical scavenging activity were selected to investigate of the liver cells protective effect against H₂O₂ (20). The liver cell protective effect of sulfated hetero-chitosan and sulfated hetero-COS from chitin was determined with sub-G1 analysis by a flow cytometer. The cells were treated with the sample prior 1 mM H₂O₂ for 24 hr. There was a little effect in the SC and sulfated SCOS I. In the SCOS III, the percentages of apoptotic cells were 46.01, 42.24, 30.86 and 22.54% at 0.125, 0.25, 0.5 and 1 mg/mL, respectively (Fig. 1). The SCOS III, which had the highest inhibitory cell apoptosis against H₂O₂—induced cell death (Fig. 1), was further subjected to the second FACS. The SCOS III clearly reduced apoptosis in dose dependent manners, as shown in Fig. 2. The chang cells were incubated with increasing concentrations of the SCOS III for 24 h. The percentages of apoptotic cells were 50.17% at 1 mM H₂O₂ treated cells (Fig. 2).