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Protective effects of aminoethyl-chitooligosaccharides against oxidative stress in mouse macrophage RAW 264.7 cells

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ABSTRACT

The aim of this study is to investigate the inhibitory effects of aminoethyl-chitooligosaccharides (AE-COS) on oxidative stress in mouse macrophages (RAW 264.7 cells). The inhibitory effects of AE-COS on DNA and protein oxidation were studied in RAW 264.7 cells. Furthermore, free radical scavenging effect of AE-COS were determined in RAW264.7 cells by 2′,7′-dichlorofluorescein (DCF) intensity and intracellular glutathione (GSH) level. AE-COS also inhibited myeloperoxidase (MPO) activity in human myeloid cells (HL-60). These results suggest that AE-COS acts as a potential free radical scavenger in RAW 264.7 cells.

1. Introduction

In biological systems, equilibrium between oxidants formation and endogenous antioxidant defense mechanisms exists to protect cellular biomolecules against oxidation. The oxidative stress will happen if that balance is disturbed [1]. Oxidative stress causes injury to important cellular components, thus the reactive oxygen species (ROS) generated excessively in tissues can lead to death of cells. Furthermore, ROS play an important role in many diseases such as cancer, arthritis, neurodegenerative, diabetes, hypertension, inflammation and aging. They have direct or indirect relationship with oxidation of cellular biomolecules [2,3]. Therefore, the studies to develop novel antioxidant are necessary.

Chitosan is prepared with deacetylation of chitin, a polysaccharide abundantly found in nature in the presence of alkali at high temperature. It has numerous biological activities such as immuno-enhancing activity, antitumor activity [4], antibacterial activity [5], antifungal activity [6], anti-hypertensive effects, and antioxidant activity [7–9]. Chitooligosaccharides (COS), partially hydrolyzed products of chitosan, is of great interest in pharmaceutical and medicinal applications due to their non-cytotoxic and high water soluble properties [7]. Moreover, the structure and properties of chitosan and its derivatives have been studied more and more. These reports helped to improve the structural properties of chitosan and the similar structural compounds for a particular application by chemical modification. However, research on synthesis of COS derivatives and identification of their biological activities have been seldom reported. Therefore, our objective was to develop a COS derivative, aminoethyl-chitooligosaccharides (AE-COS), with improved antioxidant activity in mouse macrophage RAW 264.7 cells.

2. Materials and methods

2.1. Materials

Chitooligosaccharides (MW 800–3000 Da, degree of deacetylation, DD, 90%) prepared from crab shells were donated by Kitto Life Co. (Seoul, Korea). MTT reagent (3-(4,5-dimethyl-2-yl)-2,5-diphenyltetrazolium bromide), 3,3′,5,5′-tetramethybenzidine (TMB), 2,4-dinitro-phenyl hydrazine reagent, 2′,7′-dichlorofluorescein diacetate (DCFH-DA), agarose, and fetal bovine serum (FBS) and some other chemicals were purchased from Sigma Chemical Co. (St. Louis, MO, USA).

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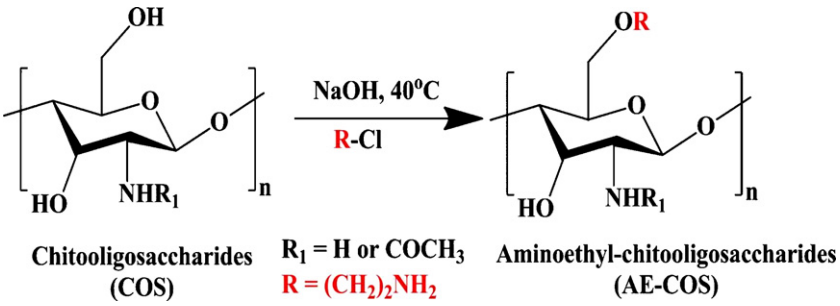

Scheme 1. Synthesis pathway of aminoethyl-chitooligosaccharides (AE-COS).

Human myeloid (HL-60) and RAW 264.7 cells were obtained from American Type Culture Collection (Manassas, VA, USA). Roswell Park Memorial Institute 1640 (RPMI-1640) and Dulbecco's Modified Eagle Medium (DMEM) medium, penicillin/streptomycin, and the other materials required for culturing of cells were purchased from Gibco BRL, Life Technologies (USA). All other chemicals were of analytical grade or of the highest grade available commercially.

2.2. Synthesis of aminoethyl-chitooligosaccharides (AE-COS)

Aminoethyl-chitooligosaccharides (AE-COS) was prepared by our previous method [10]. Briefly, aqueous 3.0 M (20 ml) 2-chlorethylamino hydrochloride was added to COS (0.40 g) while stirring at 40 °C. NaOH (3.0 M, 20 ml) was added to the reaction mixture drop wise, and continuously stirred for 48 h as in Scheme 1. After reaction, the solution was filtered using a filter paper. Subsequently, the reaction mixture was acidified with 0.1 N HCl, and dialyzed against water for 2 days. The product was freeze dried to give AE-COS (0.334 g).

AE-COS and COS were characterized by IR and ^{1}H NMR. The spectra of sample in the forms of KBr disk were obtained using a Fourier transform infrared (FT-IR) spectrometer (Perkin Elmer Spectrum GX, Beaconsfield Bucks, England) with a frequency range of 4000–400 cm^{-1}. ^{1}H NMR measurements were performed on a JEOL JNM ECP-400 NMR spectrometer under a static magnetic field of 400 MHz and chemical shift values are given in y (ppm) and its degree of substitution was calculated using C, N, and H elemental analysis (Elementar Vario, EL, USA).

2.3. Assessment of cell viability determination using the MTT assay

Cytotoxicity levels of AE-COS cell lines were measured using the MTT method as described by Hansen et al. [11]. The cells were grown in 96-well plates at a density of 5×10^3 cells/well. After 24 h, cells were washed with fresh medium and were treated with different concentrations of AE-COS. After 48 h of incubation, cells were rewashed and 50 μl of MTT (5 mg/ml) was added and incubated for 4 h. Finally, 200 μl of DMSO was added to solubilize the formazan salt formed and amount of formazan salt was determined by measuring the optical density (OD) at 540 nm using an GENios microplate reader (TECAN Austria GmbH, Austria). Relative cell viability was calculated compared to the non-treated blank group.

2.4. Myeloperoxidase activity assay

Determination of myeloperoxidase (MPO) released to HL-60, cells were treated with different concentrations of AE-COS or COS and blank (no inhibitor) an assay mixture containing 2 mM H_2O_2 and 1.6 mM 3,3′,5,5′-tetramethybenzidine (TMB) was added and amount of MPO released was measured by spectrophotometer at 655 nm [12].

2.5. Protein oxidation assay

The oxidation degree of cell proteins was assessed by determining the content of protein by carbonyl group [13]. Cultured cells were washed three times with PBS and lysed in lysis buffer without reducing agents. Aliquots of cell lysate were transferred

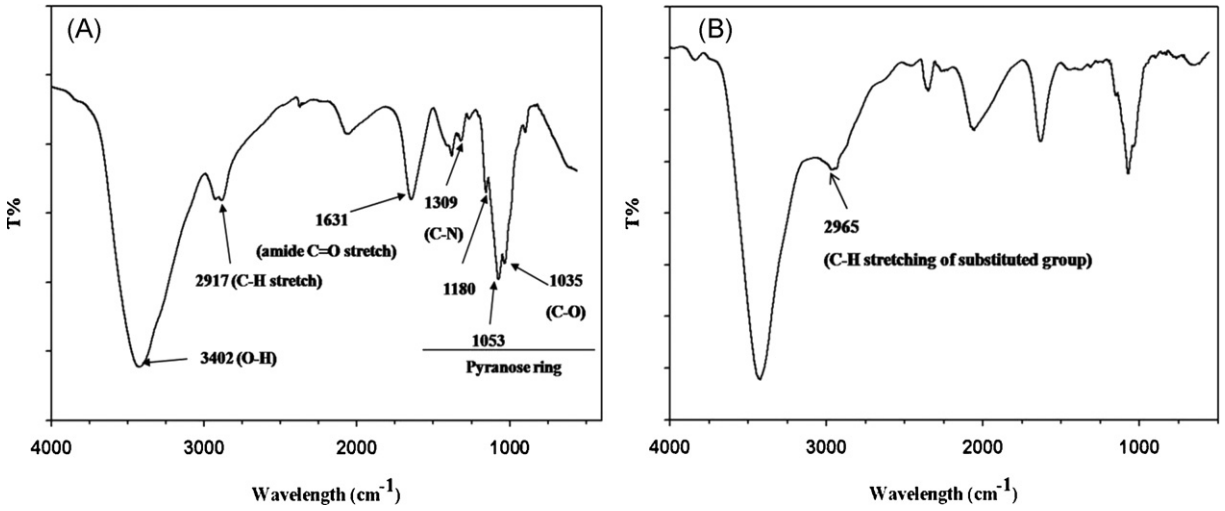

Fig. 1. FT-IR (KBr) spectra of COS (A) and AE-COS (B); ^{1}H NMR (400 MHz, D_2O) spectra of COS (C) and AE-COS (D).