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Preparation of a series of chitooligomers and their effect on hepatocytes

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ABSTRACT

A series of chitooligomers with molecular weight ranging from 1.7 ~ 3.8 × 10^3 were prepared by degradation of a high molecular weight chitosan with hydrogen peroxide and selective precipitation in ethanol solutions. The prepared chitooligomers were characterized by gel permeation chromatography, elemental analysis, Fourier transform infrared spectra, ^{13}C nuclear magnetic resonance spectroscopy and X-ray diffraction analysis. Cell culture experiments suggested that the effect of the chitooligomers on the proliferation of L02 hepatocytes was dependent on culture time, namely, at the initial stage of culture there was an inhibitory effect on proliferation of the cells; however, the cultures recovered in cell proliferation and exhibited promotion effect in following days. In the case of chitosan monomer (GlcN), high concentration of GlcN (1 mg/ml) produced a significant suppression in proliferation of L02 cells relative to control, with decreases of 35.2%, 60% and 72.9% on days 1, 3 and 5, respectively. In addition, there was no significant effect of the chitooligomers on the functions of albumin secretion and urea synthesis of the hepatocytes.

1. Introduction

Chitooligomers, a class of chitosans with degree of polymerization <20, are known to have some special biological activities such as antibacterial activity (Jeon, Park, & Kim, 2001), antitumor and immune enhancing effects (Jeon & Kim, 2002; Qin, Du, Xiao, Li, & Gao, 2002; Tokoro et al., 1988). These functions depend not only on their chemical structure but also on the molecular size. In recent years, studies of the effect of chitooligomers on various cells, e.g. polymorphonuclear cells (Usami, Okamoto, Takayama, Shigemasa, & Minami, 1998), macrophages (Okamoto et al., 2003), fibroblasts (Mori et al., 1997), endothelium (Okamoto et al., 2002), osteoblasts (Ohara et al., 2004) and red blood cells (Fernandes et al., 2008), have attracted more and more interest. The study of behaves of polymorphonuclear cells (PMNs), macrophages, endothelium and fibroblasts treated with chitooligomers have been related with the mechanisms of induction of acceleration of wound healing by chitin and chitosan. It has been demonstrated chitooligomers induced chemotactic migration of PMNs and stimulated the induction of interleukin-8 of fibroblast (Mori et al., 1997; Usami et al., 1998). However, to our best knowledge, there is no report on the effect of chitooligomers on hepatocytes.

Chitooligomers can be obtained by enzymatic depolymerization of chitosan (Qin, Wang, Peng, Hu, & Li, 2008) or by chemical depolymerization with acids such as hydrochloric acid (Belamie, Domard, & Giraud-Guille, 1997), nitric acid (Tommeraas, Varum, Christensen, & Smidrod, 2001) and sulfuric acid (Nagasawa, Tohira, Inoue, & Tanoura, 1971). In general, enzymatic methods have advantages over chemical reaction, since enzymes operate under milder conditions and are highly specific; however, their commercial use is limited due to cost and limited availability (Li et al., 2005). In addition, chitooligomers can also be obtained by oxidative depolymerization with oxidants, such as ozone (Yue, He, Yao, & Wei, 2009), sodium nitrite (Allan & Peyron, 1995) and hydrogen peroxide (Chang, Tai, & Cheng, 2001; Qin, Du, & Xiao, 2002; Tian, Liu, Hu, & Zhao, 2004; Wang, Huang, & Wang, 2005). Hydrogen peroxide has long been used in the treatment of chitosan because it is easy to handle, easily available and environmentally friendly. In particular, for cell culture applications, the method does not cause impurities in products. However, it was reported the reaction occurred in a random pattern, resulting in a broad distribution of molecular weight of the chitooligomers (Qin, Du, & Xiao, 2002).

The goal of this work was to prepare a series of chitooligomers with different of molecular weights, and to explore preliminarily the effect of prepared chitooligomers on the hepatocytes. Here, we describe a quick and simple method for the production of a homogeneous series of chitooligomers, varying in molecular weight from 1.7 to 3.8 × 10^3, with low polydispersity. The prepared method involves a simple chemical process and only selective precipitations. Finally, cell proliferation, morphology and functions such as albumin secretion and urea synthesis were employed to determine the effect of the chitooligomers on the hepatocytes.

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2. Materials and methods

2.1. Materials

Original chitosan (CS0) with a degree of deacetylation (DD) of 91% was purchased from Zhejiang Aoxing Biotechnology Co., Ltd. (Zhejiang, China). Its viscosity average molecular weight is about 300,000. Chitosan monomer (D-glucosamine hydrochloride), penicillin and streptomycin were supplied by Solarbio (Beijing, China). RPMI-1640 was obtained from Gibco (USA). Fetal bovine serum (FBS) was purchased from HyClone (USA). Urea Nitrogen Kit and Albumin Detection Kit were from Nanjing Jiancheng Bioengineering Institute (Nanjing, China). All other chemicals were of analytical grade and were used without further purification.

2.2. Preparation of a series of chitooligomers

CS0 (20 g) was completely dissolved in 800 ml 2% (w/v) acetic acid with a water bath at 70 °C, then 30 ml 30% (v/v) hydrogen peroxide was added to the solution. The resulting solution was stirred and reacted for 2 h. After the reaction, the solution was immersed in an ice bath and neutralized to pH 8.0 with concentrated NaOH to remove high molecular weight chitosan by precipitation. The filtered solution was precipitated with final ethanol concentrations of 50%, 75% and 87.5% (v/v). Each precipitate was centrifuged, washed with ethanol and vacuum dried. COS1, COS2 and COS3 are the products of selectively precipitation with final ethanol concentrations of 50%, 75% and 87.5% (v/v), respectively.

2.3. Characterization

Weight average molecular weight (M_w), number average molecular weight (M_n) and molecular weight dispersion (M_w/M_n) were determined by a gel permeation chromatography (GPC) equipped with a Waters 515 HPLC pump, a Waters 2410 refractive index detector and a OHpak KB-803 HQ column operated at a flow rate of 0.7 ml/min. Each sample with a volume of 10 μl (5 mg/ml) was eluted with a solution of 0.5 M CH_3COOH/0.5 M CH_3COONa at 25 °C. Dextranum standards (National Institute for The Control of Pharmaceutical and Biological Products, China) were used for column calibration and as a relative reference for molecular weight calculation. All data were collected and analyzed using the Empower software.

The elemental analyses were performed with a Vario EL III elemental analyzer. The DD of the sample was calculated by the following equation (Tian et al., 2004; Xu, McCarthy, & Gross, 1996):

$$\mathrm{DD} = \left(1 - \frac{\mathrm{C/N} - 5.14}{1.72}\right) \times 100\%$$

where C/N is the ratio (w/w) of carbon to nitrogen.

Fourier transform infrared spectra (FT-IR) were recorded with KBr pellets on a Nicolet 670 FT-IR Spectrometer.

^{13}C nuclear magnetic resonance spectroscopy (^{13}C NMR) analyses were recorded on a Bruker AV II-400 MHz spectrometer. The products were dissolved in D_2O in the presence of CF_3COOD.

X-ray diffraction (XRD) measurements were carried out on an X' Pert Pro MPD X-ray diffractometer (Philips, Netherlands) and used a Cu Kα target at 40 kV and 40 mA at 25 °C.

2.4. Preparation of chitooligomer solution

Each chitooligomers and chitosan monomer (D-glucosamine hydrochloride) were dissolved with PBS at a concentration of 10 mg/ml. Completely dissolved solution was neutralized to pH ~7.2, and then sterilized with a 0.2 μm filter.

2.5. Cell culture

Normal human hepatocytes, L02, obtained from Type Culture Collection of Chinese Academy of Sciences (Shanghai, China) were used for cell assay. L02 hepatocytes were grown and maintained in RPMI-1640 medium supplemented with 10% FBS, 100 μg/ml penicillin and 100 μg/ml streptomycin at 37 °C in a 5% CO2 atmosphere, with medium changed every day until confluent cell monolayer was formed. The cells in their three passages were used in experiments.

2.6. Cell proliferation assay

L02 hepatocytes were adjusted to a concentration of 5×10^4 cells/ml in the culture medium before the experiment. The cells were seeded in 96 well plates at a density of 5×10^3 cells/well. RPMI-1640 containing 10% FBS, 100 μg/ml penicillin and 100 μg/ml streptomycin in supplement with 1 mg/ml chitooligomer was prepared using the 10 mg/ml chitooligomer solution. This mixture solution was diluted by 10-fold steps with above RPMI-1640 medium to prepare 0.1 and 0.01 mg/ml solutions. 1, 0.1 and 0.01 mg/ml chitosan monomer solutions were also prepared according to above method. After 2 days, the seeded cells were washed with PBS and then treated with different concentrations of chitooligomers or chitosan monomer. As a control, PBS was added to media in place of chitooligomers or chitosan monomer suspension.

The cell proliferation was analyzed by using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT, Sigma) mitochondrial reduction. This assay is based on the ability of live cells to reduce a tetrazolium-based compound, MTT, to a purplish formazan product. After 1, 3 and 5 days of culture, the cells were observed under a phase contrast microscope (CKX41, Olympus, Japan) before adding 20 μl of MTT solution (5 mg/ml) to each well and the cells were incubated at 37 °C for 4 h. After removing the culture media, 150 μl of DMSO was added, and the plates were shaken for 10 min. The optical density (OD) of each well was determined using a microplate reader at a wavelength of 490 nm. The OD value is proportionate to the cell numbers.

2.7. Albumin synthesis determination

The L02 cells with a density of 5×10^4 cells/ml were seeded in 6 well plates in RPMI-1640 supplemented with 10% FBS, 100 μg/ml penicillin and 100 μg/ml streptomycin at 37 °C in a 5% CO2 atmosphere. After the cells formed confluent layers, the culture medium was discarded and replaced with the fresh medium containing chitooligomers or chitosan monomer with a concentration of 0.1 mg/ml. The medium was refreshed daily and the collected medium was centrifuged in 14,000 rpm for 10 min. The supernatant was stored at −20 °C for albumin assay. The concentration of albumin remaining in the supernatant was measured by the bromocresol-green method which based on an established method that bromocresol-green forms a coloured complex specifically with albumin (Doumas, Watson, & Biggs, 1971). Levels of albumin were determined using a commercially available kit (Albumin Detection Kit). Known quantities of human albumin were used to establish the standard curve.

2.8. Urea synthesis assay

To assess the urea synthesis function of the L02 cells treated with chitooligomers, the culture medium was replaced with fresh medium containing 5 mm NH_4Cl. The cells were cultured in this medium for 120 min, before the medium was again replaced with the normal medium. The collected medium was tested for urea production using a Urea Nitrogen Kit.