J. Microbiol. Biotechnol. (2007), **17**(9), 1546–1549

JOURNAL OF MICROBIOLOGY AND BIOTECHNOLOGY

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Chemopreventive Effect of Chitosan Oligosaccharide Against Colon Carcinogenesis

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Received: June 5, 2007 Accepted: July 11, 2007

Abstract Chitosan oligosaccharide (COS, 3 kDa<MW<5 kDa) was tested for colon cancer chemoprevention by measuring the activities of quinine reductase (QR) and glutathione-*S*-transferase (GST), glutathione (GSH) levels, ornithine decarboxylase (ODC) activity, and cyclooxygenase (COX)-2 expression in HT-29 cells treated with COS. COS induced QR activity in a dose-dependent manner over a concentration range of 0.1–4.0 mg/ml. GST activity was also induced in HT-29 cells treated with COS. In addition, GSH levels were increased 1.3-, 1.4-, and 1.5-fold with COS at 2, 3, and 4 mg/ml, respectively. ODC activity induced by 12-*O*-tetradecanoylphorbol-13-acetate (TPA) was inhibited by 33% and 39% with 3 and 4 mg/ml of COS, respectively. COS also inhibited the expression of TPA-induced COX-2 protein in HT-29 cells. These results suggest that COS has colon cancer chemopreventive activity by increasing QR and GST activities and GSH levels and by inhibiting ODC activity and COX-2 expression *in vitro*.

Keywords: Chitosan oligosaccharide, colon cancer, cyclooxygenase, glutathione *S*-transferase, ornithine decarboxylase, quinone reductase

Colorectal cancer is one of the most common cancers in the industrialized world and is a major cause of morbidity and mortality [12]. Cancer risk is associated with both inherent factors and environmental factors, including diet. Interestingly, dietary factors play an important role in influencing human colon cancer risk. Epidemiological and laboratory studies indicate that high consumption of fruits and vegetables leads to reduced incidence of colorectal cancer [3, 6]. Thus, colorectal cancer is an ideal disease in which to evaluate the potential benefits of chemopreventive agents.

Chemopreventive agents can function through a variety of mechanisms throughout all major stages of carcinogenesis. One mechanism of particular note involves the induction of detoxification enzymes such as quinone reductase (QR) and glutathione *S*-transferase (GST) [21]. Detoxification enzymes have been investigated as biomarkers of risk for various cancers including colorectal cancer. QR is a major enzyme of xenobiotic metabolism and protects cells against mutagenicity and carcinogenicity. GST detoxifies a number of carcinogenic electrophiles by catalyzing conjugation with glutathione (GSH). A major mechanism by which GST inactivates chemical carcinogens could be its blocking the formation of DNA adducts [15].

GSH plays a role in the detoxification of a variety of endogenous and exogenous electrophilic compounds and peroxides after catalysis by GST. Therefore, GSH may protect cells against toxicity arising from exposure to excessive amounts of electrophiles.

Ornithine decarboxylase (ODC) is a rate-limiting enzyme in the polyamine biosynthetic pathway [9]. ODC activity has been found to be significantly increased in tumors compared with nontumor tissues [14]. Therefore, ODC activity may be a useful biomarker of tumor growth rate and biological aggressiveness.

Cyclooxygenase (COX) is a key enzyme in the biosynthesis of prostaglandin from arachidonic acid. Two isoforms, COX-1 and COX-2, have been identified. COX-2 is induced by certain serum factors, growth factors, proinflammatory cytokines, and tumor promoters [22]. There are reports that COX-2 is highly expressed in colon tumors [2]. COX-2 inhibitors are effective in preventing colon cancer in several animal models [10].

Crab and shrimp shell wastes are utilized as the major industrial source of biomass for the large-scale production of chitosan oligosaccharides (COS). The water-soluble COS possess various biological activities, including antitumor activity and immunoenhancing effects [11, 20], and have been shown to be particularly useful in many fields [7, 16].

In this study, the effects of COS on induction of QR and GST activities and GSH levels, and inhibition

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of ODC activity and COX-2 protein expression were investigated.

MATERIALS AND METHODS

Preparation of Chitosan Oligosaccharide

Water-soluble chitosan oligosaccharide (3 kDa<MW<5 kDa) was prepared from 1% (w/v) chitosan in a dual reactor system [1]. Chitosan (1%, w/v) was dissolved in 0.27 M lactic acid, and the pH of the solution was adjusted to 5.5 with $NaHCO_3$. The dual reactor system was composed of a column reactor packed with immobilized chitosanase (derived from *Bacillus pumilus* BN-262) and an ultrafiltration membrane reactor (Millipore Minitan system, molecular weight cutoff 5,000, and 3,000 membrane) [23]. Chitosan was partially hydrolyzed using the packed column reactor, and the partially hydrolyzed chitosan was then supplied to a substrate feed tank with an ultrafiltration membrane reactor to produce chitosan oligosaccharide.

Cell Culture

Human colon adenocarcinoma cell line, HT-29, was obtained from the Korean Cell Line Bank (Seoul, Korea). It was cultured in RPMI-1640 medium supplemented with 10% fetal bovine serum.

QR and GST Activities Assays

QR activity was measured in HT-29 human colon adenoma cells grown in 96-well microtiter plates, according to the method of Shon *et al.* [19]. The increase in QR activity was calculated from the ratio of the specific enzyme activity in sample-treated cells compared with a solvent-treated control.

GST activity in HT-29 cells was measured at 380 nm with 1-chloro-2,4-dinitrobenzene (CDNB) as the substrate according to the procedure developed by Habig *et al.* [4] with modification. The protein content was measured in a duplicate plate using a bicinchoninic acid protein assay kit (Sigma, St. Louis, MO, U.S.A.) with bovine serum albumin as the standard. The GST activity was expressed as the slope/min/mg protein. The data derived from sample-treated cells were compared with the values obtained for solvent-treated controls. Ellagic acid was used as a positive control.

GSH Content Assay

GSH levels in HT-29 cells were determined using the glutathione reductase-coupled 5,5'-dithiobis 2-nitrobenzoic acid (DTBN) enzyme-linked immunosorbent assay (ELISA) in 96-well format [13].

ODC Activity Assay

The ODC activity in HT-29 cells was evaluated according to the method of Shon and Nam [18] by measuring the release of $^{14}CO_2$ from L-[1-^{14}C] ornithine.

COX-2 Expression (Western Blotting)

HT-29 cells were plated in 12-well plates at a density of 3×10^5 cells/well and treated with various concentrations of COS (0.1, 0.5, 1, 2, 3, and 4 mg/ml) in the presence of 200 nM 12-*O*-tetradecanoylphorbol-13-acetate (TPA). Confluent cells were washed twice with cold phosphate-buffered saline (PBS), lysed in lysis buffer [10 mM HEPES, 10 mM HCl, 0.1 mM EDTA (pH 8.0), 0.1 mM EGTA, 0.5 mM PMSF, 1 mM DTT, and 10% Nonidet P40], and then clarified by centrifugation (12,000 rpm, 10 min) at 4°C. The cell lysate proteins (25 μg) were electrophoresed on 9% SDS-polyacrylamide gels and then electrophoretically transferred to polyvinylidene difluoride membranes using the Hoefer electrotransfer system (Amersham Bioscience, Amersham, U.K.). The membranes were incubated with 5% nonfat milk overnight to block nonspecific binding. To detect COX-2, the membranes were incubated with mouse anti-COX-2 antibody (1:3,000) for 2 h at room temperature. The membranes were then incubated with biotin-rabbit anti-mouse Ig GAM (H+L) and alkaline phosphate-conjugated streptavidin and visualized with the 4-nitroblue tetrazolium chloride/5-bromo-4-chloro-3-indolyl-phosphate substrate (Promega, Madison, WI, U.S.A.).

Statistical Analysis

The data were analyzed for statistical significance using Student's *t*-test. p values less than 0.05 were considered significant.

RESULTS AND DISCUSSION

Effect of COS on QR and GST Activities

The effect of COS on QR and GST activities in HT-29 human colon cancer cells was analyzed. COS dose-dependently increased QR activity over the concentration range of 0.1–4.0 mg/ml (Fig. 1).

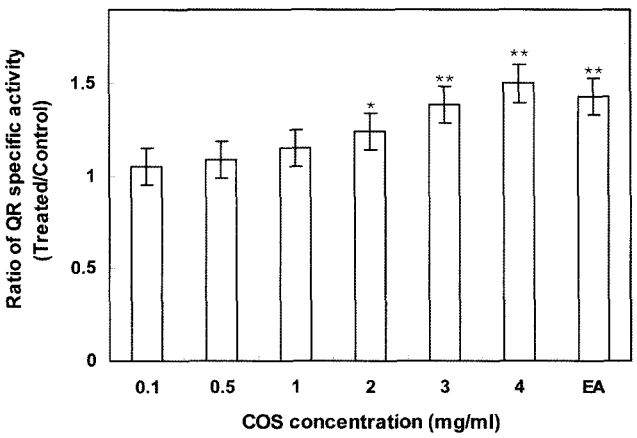

Fig. 1. Effect of chitosan oligosaccharide on QR activity.
EA, 15 μg/ml ellagic acid. Data shown are mean values with bars indicating the SD of the mean (n=3). *p<0.05, **p<0.01 *vs.* control.

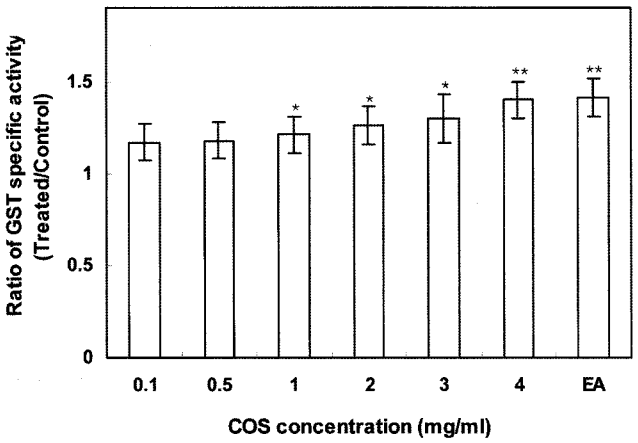

Fig. 2. Effect of chitosan oligosaccharide on GST activity.
EA, 15 μg/ml ellagic acid. Data shown are mean values with bars indicating the SD of the mean (n=3). *p<0.05, **p<0.01 *vs.* control.

GST activity, as measured with CDNB, was also increased over a concentration range of 0.1–4.0 mg/ml with a maximal 1.4-fold induction at the highest concentration (Fig. 2).

Detoxification enzymes play an important role in preventing carcinogen-induced colon cancer [5] and are surrogate biomarkers for chemopreventive potential. In the current study, we observed that COS treatment increased the activities of the detoxification enzymes QR and GST in human colon cancer HT-29 cells. Thus, COS may prevent the initiation of colon carcinogenesis by increasing phase II enzyme activity.

Effect of COS on GSH Levels

To investigate the potential of COS to induce GSH levels, cells were treated with various concentrations of COS (0.1–4.0 mg/ml). The results indicate that GSH levels were increased 1.3-, 1.4-, and 1.5-fold with 2 (p<0.05), 3 (p<0.01), and 4 mg/ml (p<0.01) COS, respectively.

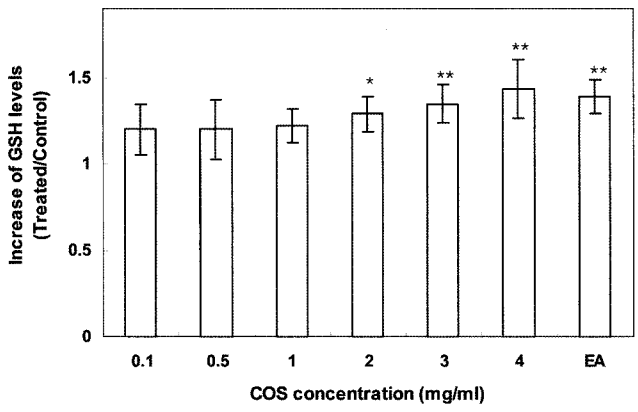

Fig. 3. Effect of chitosan oligosaccharide on GSH levels.
EA, 15 μg/ml ellagic acid. Data shown are mean values with bars indicating the SD of the mean (n=3). *p<0.05, **p<0.01 *vs.* control.

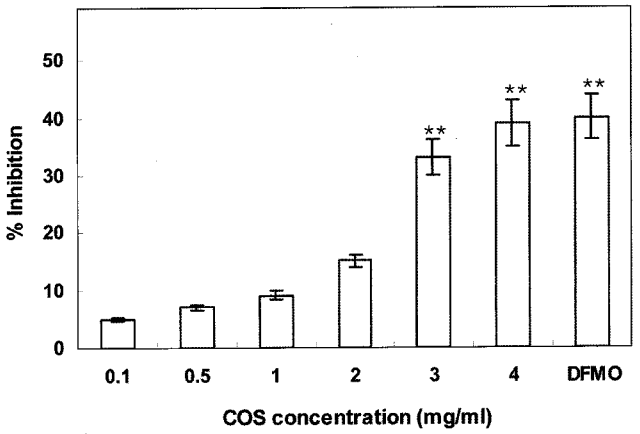

Fig. 4. Effect of chitosan oligosaccharides on TPA-induced ODC activity in HT-29 human colon adenoma cells.
The control ODC activity is 512±47 pmol $^{14}CO_2$/h/mg protein. DFMO, 0.01 mM difluoromethylornithine. Data shown are mean values with bars indicating the SD of the mean (n=3). **p<0.01 *vs.* control.

GSH is important in the detoxification of a variety of electrophilic compounds and peroxides after its catalysis by GST, and induction of GSH is used to test potential chemopreventive agents [17]. Therefore, enhancement of GSH by COS may decrease or inhibit carcinogenesis.

Effect of COS on ODC Activity

The effect of COS on ODC activity was examined. As shown in Fig. 4, treatment of HT-29 cells with COS inhibited TPA-induced ODC activity in a dose-dependent manner up to 4 mg/ml. At 3 and 4 mg/ml (p<0.01), COS treatment resulted in 33% and 39% inhibition of TPA-induced ODC activity, respectively. A comparable effect was observed with difluoromethylornithine (DFMO), a suicide inhibitor of ODC. ODC has been a major target for anticancer investigations. Excess induction of ODC activity stimulates tumor promotion. Inhibition of ODC activity may be one of several antitumor-promoting effects of COS in colon cancer development.

Effect of COS on COX-2 Expression

The effect of COS on COX-2 protein expression in HT-29 cells was examined by Western blot analysis. As shown

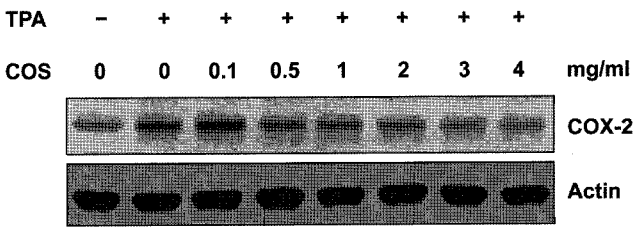

Fig. 5. Effect of chitosan oligosaccharides on the TPA-induced protein expression of COX-2.

in Fig. 5, COS inhibited the TPA-induced expression of COX-2 protein. There is increasing evidence suggesting that COX-2 inhibitors can be effective as antiinflammatory agents and also in the prevention and treatment of colon cancer. Since prostaglandins are mediators of inflammation and chronic inflammation predisposes to carcinogenesis [8], agents that can inhibit COX-2 might therefore be useful for the inhibition of colon carcinogenesis.

The results of the current study suggest that COS may act as a colon cancer chemopreventive agent because of its specific activity for the induction of QR and GST activities and GSH levels and inhibition of ODC activity and COX-2 expression. Therefore, the current data may provide useful information for the further development of COS as a colon cancer chemopreventive agent in animal studies and in subsequent human clinical trials.

Acknowledgment

This research was supported by a grant (M2007-08) from the Marine Bioprocess Research Center of the Marine Bio 21 Center funded by the Ministry of Maritime Affairs & Fisheries, Republic of Korea.

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