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RESEARCH NOTE

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Cytotoxic Effects of Partially Purified Substances from *Bacillus polyfermenticus* SCD Supernatant toward a Variety of Tumor Cell Lines

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Abstract The cytotoxic effects of partially purified substances from *Bacillus polyfermenticus* SCD toward a variety tumor cell lines were studied. Cytotoxic activity was determined with regard to the A549 (human lung carcinoma), AGS (human stomach adenocarcinoma), DLD-1 (human colon adenocarcinoma), HEC-1-B (human uterus adenocarcinoma), SW-156 (human kidney carcinoma), and NIH/3T3 (murine normal fibroblast) cell lines using the MTT assay. Cytotoxic substances were partially purified through Diaion HP-20 columns and extracted with methanol or other organic solvents (*n*-hexane, chloroform, ethylacetate, and butanol). *B. polyfermenticus* SCD supernatant showed up to 60% inhibition of cell viability for all five human cancer cell lines tested. When treated with 10 mg/mL of *n*-hexane, chloroform, ethylacetate, and butanol extract, HEC-1-B cells showed a 25, 62, 35, and 63% rate of inhibition, respectively, and AGS cells showed a 72, 61, 44, and 67% rate of inhibition, respectively. At a concentration of 10 mg/mL, 100% methanol Diaion HP-20 extracts showed inhibition rates of 97.0% toward A-549 cells, 98.1% toward AGS cells, 81.6% toward DLD-1 cells, 83.5% toward HEC-1-B cells, and 92.7% toward SW-156 cells. These results indicate that partially purified fractions from *B. polyfermenticus* SCD have the potential to inhibit not only colon cancer cells, but also lung, stomach, uterus, and kidney cancer cells. Further studies are needed to characterize the cytotoxic substances released in *B. polyfermenticus* SCD cultures.

Keywords: *Bacillus polyfermenticus* SCD, cytotoxic effect, MTT assay, Diaion HP-20

Introduction

Beneficial bacteria, which have been used for medical purposes in the treatment of intestinal disorders, include strains of *Bifidobacterium, Lactobacillus, Enterococcus, Clostridium butyricum, Bacillus subtilis,* and *Bacillus polyfermenticus*. In particular, strains of *B. polyfermenticus*, commonly known as 'Bispan' strains, have been used for the treatment of long-term intestinal disorders, since the live strains in the form of active endospores can reach the target intestine (1).

B. polyfermenticus strains are described in the Japanese Pharmacopoeia as amylolytic bacilli, together with *B. subtilis* and *Bacillus mesentericus*. However, the term Bispan does not appear in the international nomenclature such as Bergey's Manual of Systematic Bacteriology (2). *B. polyfermenticus* SCD is distinct from *B. subtilis* strains in being able to metabolize lactose and produce a larger amount of acetic and lactic acid from glucose and lactose, respectively.

Cancer is one of the major chronic diseases worldwide, claiming over seven million deaths each year. Despite significant advances in cancer treatment, cancer has not yet been fully conquered. Much attention has recently been given to cancer prevention, i.e., chemoprevention via the ingestion of chemical substances (natural or synthetic) that reduce the risk of carcinogenesis (3). At present, the MTT assay using human and other tumor cell lines is one of the most widely used methods to evaluate the cytotoxicity of various extracts and active constituents (4, 5). A number of studies in animal models and in human populations have demonstrated that the consumption of probiotic bacteria can reduce the risk of colon cancer. Most of these studies have focused on lactic acid bacteria (LAB) such as *Lactobacillus* spp. and *Bifidobacterium* spp. (6, 10). The cytotoxic effects of *B. polyfermenticus* strains have not yet been reported, despite their beneficial effects on health. In a recent *in vitro* study, Park *et al.* (7) determined that *B. polyfermenticus* SCD supernatant has inhibitory activity toward human colon cancer cells (Caco-2).

The goal of this study was to determine the cytotoxic activity of partially purified substances from *B. polyfermenticus* SCD supernatant toward various human cancer cell lines using the MTT assay.

Materials and Methods

Bacterial strain and culture conditions *B. polyfermenticus* SCD was stored at -70°C in tryptic soy broth (TSB; Difco Laboratories, Detroit, MI, USA) containing 20%(v/v) glycerol. Cultures were grown in 500 mL of TSB medium in 1,000 mL flasks. The temperature was maintained at 37 °C and the agitation speed was 150 rpm.

Partial purification of *B. polyfermenticus* SCD supernatant Culture broth was centrifuged at 15,000×g for 20 min at 4°C and the supernatant was then sterilized though a 0.22 μm pore size syringe filter. The sterile filtered supernatant was injected into a 5 cm (diameter) × 1 m (length) Diaion HP-20 column at a ratio of 1:2 (volume of Diaion HP-20 resin:supernatant). Substances were eluted with a stepwise gradient of water-methanol (0, 20, 40, 60, 80, and 100% of methanol) in the mobile phase at a 15 mL/min flow rate. Each fraction was concentrated using a rotary vacuum evaporator.

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Extraction with nonpolar solvents The supernatant of *B. polyfermenticus* SCD cultures was also extracted with various nonpolar solvents (chloroform, *n*-hexane, ethyl acetate, or butanol) while shaking overnight at room temperature. Each fraction was concentrated using a rotary vacuum evaporator (Eyela, Tokyo, Japan).

Cell lines and culture conditions Five different human cancer cell lines and a normal murine cell line were purchased from the Korean Cell Line Bank (KCLB; Seoul National University, Seoul, Korea). A549 (human lung carcinoma), AGS (human stomach adenocarcinoma), DLD-1 (human colon adenocarcinoma), HEC-1-B (human uterus adenocarcinoma), SW-156 (human colon carcinoma), and NIH/3T3 (murine normal fibroblast) cell lines were maintained in RPMI1640 (Gibco Laboratories, Grand Island, NY, USA) medium containing 10% heat-inactivated fetal bovine serum (HyClone, Logan, UT, USA), penicillin (100 U/mL), and streptomycin (100 U/mL). Cells were cultured in a 5% CO_2 incubator at 37°C (8). For the testing of cytotoxic activity, cells were seeded in new dishes and grown to 80% confluency.

***In vitro* cytotoxicity test (MTT assay)** The *in vitro* cytotoxic effects of *B. polyfermenticus* SCD supernatant were assessed by [3-4,5-dimethylthizol-2-yl]-2,5-diphenylate-tetrazolium bromide (MTT, Sigma, St. Louis, MO, USA) assay as described by Park *et al.* (17). Briefly, 100 μL of the cell suspension (1×10^4 cells/mL) was aliquoted into 96-well microtiter plates and incubated for 24 hr. One hundred μL of *B. polyfermenticus* SCD supernatant or partially purified substances was added to each cell suspension, and then incubated at 37°C degrees for 40 hr. At the end of the incubation, MTT solution (2.5 mg MTT/mL phosphate buffered saline) was added and the plate was further incubated for 4 hr. The supernatant was then removed from each cell suspension and 100 μL of dimethylsulfoxide was added to dissolve the colored formazan crystals produced from reaction with MTT. The optical density (O.D.) values of each solution were measured with an enzyme-linked immunosorbent assay (ELISA) plate reader at 540 nm.

Results and Discussion

Cytotoxic effects of *B. polyfermenticus* SCD supernatant MTT is a tetrazolium salt which is reduced to formazan by living cells via the 'succinate-tetrazolium reductase' system. The formazan produced by the cellular suspension is directly correlated with the number of metabolically active cells, and the colorimetric MTT assay is used to assess cell proliferation. Therefore, we conducted the MTT assay to evaluate the cytotoxic effects of *B. polyfermenticus* SCD toward human cancer cells. Figure 1 shows the cytotoxic effects of *B. polyfermenticus* SCD supernatant toward five human cancer cell lines. The supernantant was cytotoxic to each human tumor cell line following exposures ranging from 6 to 12 hr. *B. polyfermenticus* SCD supernatant suppressed cell proliferation of the A549, AGS, DLD-1, HEC-1-B, and SW-156 cell lines by 60.7, 57.1, 62.5, 62.0, and 59.4%, respectively.

In addition, we examined the cytotoxic effects of *B. polyfermenticus* SCD toward a non-tumor cell line, NIH/3T3 (murine fibroblast cell line). The proliferation of normal murine fibroblast NIH/3T3 cells was affected less than the human cancer cell lines. This suggests that *B. polyfermenticus* SCD has selective cytotoxic effects and that normal fibroblasts are less sensitive to *B. polyfermenticus* SCD supernatant than malignantly proliferating stomach, lung, colon, and uterus tumor cells (12). Figure 2 shows growth inhibition in relation to the concentration of *B. polyfermenticus* SCD supernatant and tryptic soy broth of DLD-1 cells. At a supernatant concentration of 10 mg/mL or higher, the growth of DLD-1 cells was inhibited.

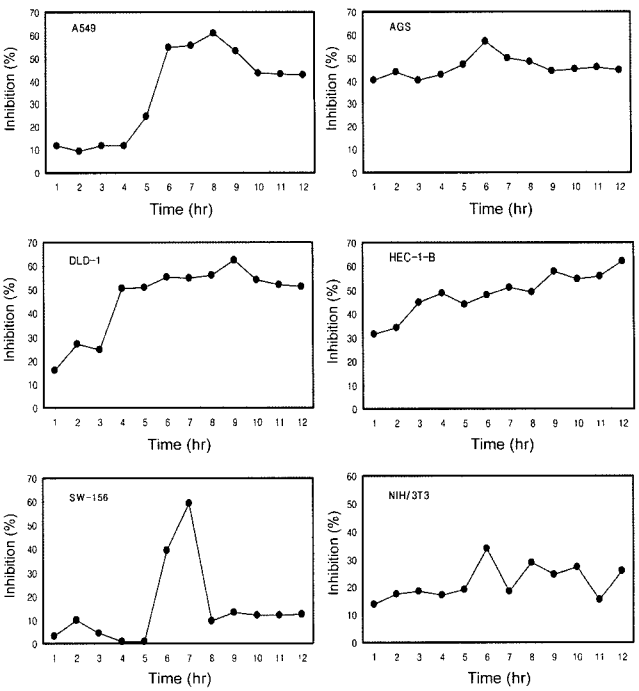

Fig. 1. Cytotoxicity of *Bacillus polyfermenticus* SCD culture supernatant toward various cell lines as a function of incubation time.

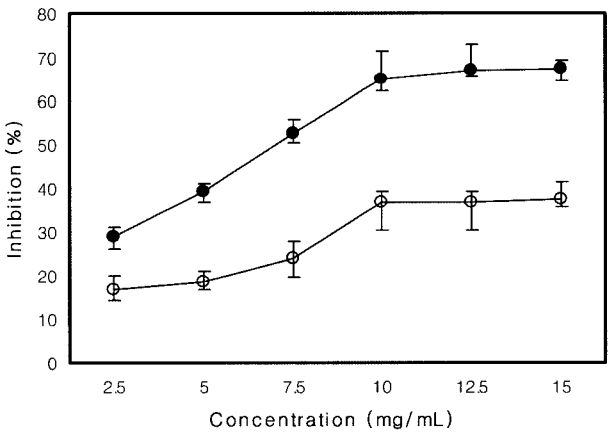

Fig. 2. Cytotoxicity of *Bacillus polyfermenticus* SCD supernatant as a function of concentration toward DLD-1 cells. ○, trytic soy broth; ●, *B. polyfermenticus* SCD supernatant.

Cytotoxic effects of fractions extracted with non-polar solvents Figure 3 shows the cytotoxic effects of *B. polyfermenticus* SCD supernatant fractions obtained using the nonpolar solvents n-hexane, chloroform, ethyl acetate, and butanol with regard to the HEC-1-B and AGS cell lines. The BuOH soluble fraction showed moderate cytotoxicity with 63 and 67% inhibition of the human colon and stomach cell lines, respectively. The degree of polarity of each solvent is *n*-hexane<chloroform<ethyl acetate<butanol<ethanol<methanol. As the extraction solvent polarity increased, the cytotoxic effects of the respective fractions increased correspondingly. This indicates that the cytotoxic components of *B. polyfermenticus* SCD supernatant are concentrated in the fractions obtained with polar solvents. However, the EtOAc soluble fraction showed no effects on either cell line.

Cytotoxic effects of Diaion HP-20 column fractions using absolute methanol Diaion HP-20 is a polymeric resin based on styrenedivinylbenzene with a mean pore size of 0.3 nm and a surface area of 500 m^2/g. This resin is widely used for a broad range of applications, including the purification of amino acids, sugars, and antibiotics (13). Figure 4 shows the cytotoxic activity of *B. polyfermenticus* SCD supernatant fractions obtained using a Diaion HP-20 column with absolute MeOH. The water and 60% MeOH extracts contained 34.8 and 65% inhibitory activity, respectively, toward the five human cancer cell lines tested. However, the absolute MeOH extract (10 mg/mL) exhibited 97.0, 98.1, 81.6, 83.5, and 92.7% inhibitory activity toward to the A549, AGS, DLD-1,HEC-1-B, and SW-156 cell lines, respectively.

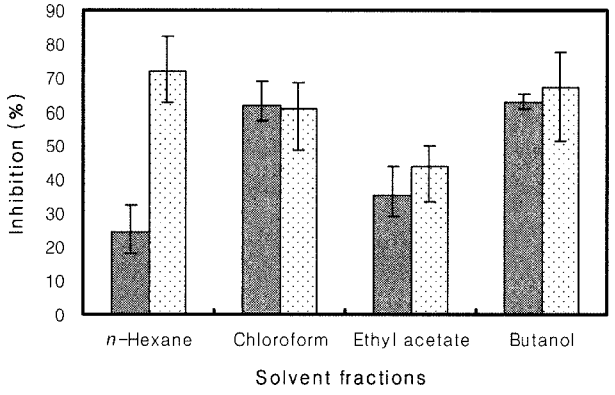

Fig. 3. Cytotoxicity of various organic solvent extracts from *Bacillus polyfermenticus* SCD supernatant. ■, HEC-1-B ; ▒, AGS

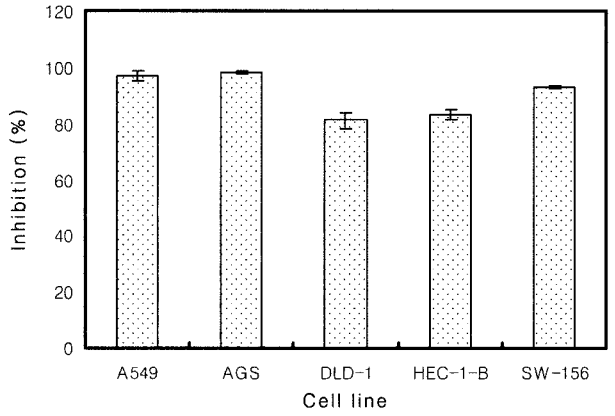

Fig. 4. Cytotoxicity of *Bacillus polyfermenticus* SCD fractions obtained by adsorption on a Diaion HP-20 column using 100 % methanol.

Baricault *et al.* (9) reported that LAB significantly reduce the growth and viability of HT-29 human colon cancer cells in culture, and that dipeptidyl peptide IV and brush border enzyme levels were significantly upregulated, suggesting that these cells may have entered a differentiation process. Although the cytotoxic action of *B. polyfermenticus* SCD or LAB is far from clearly being elucidated, the increased apoptosis of colon cancer cells, as observed in an animal study, may play a role. Another possible mechanism for the cytotoxic effects of *B. polyfermenticus* SCD may be related to the results of a recent human intervention study. Park *et al.* (21) showed that 2 weeks of *B. polyfermenticus* SCD administration to healthy adults (3.334×10^7 CFU/day) improved the fecal microflora composition by increasing the numbers of total aerobic bacteria, LAB, and bifidobacteria, and by reducing *Clostridium perfringens* and coliform strains. In addition, *B. polyfermenticus* SCD administration lowered the pH of fecal samples. These changes are likely to provide some form of protection from the development of cancer.

In conclusion, the present work shows that partially purified fractions from *B. polyfermenticus* SCD supernatant have the potential to inhibit not only colon cancer cells, but also lung, stomach, uterus, and kidney cancer cells. Further studies are needed to characterize the cytotoxic substances within these fractions.

Acknowledgments

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