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In Vitro Cancer Chemopreventive Activities of Polysaccharides from Soybeans Fermented with *Phellinus igniarius* or *Agrocybe cylindracea*

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Abstract Chemopreventive activities of polysaccharides from soybeans fermented with either *Phellinus igniarius* or *Agrocybe cylindracea* were investigated by measuring the induction of quinone reductase (QR), glutathione S-transferase (GST) activities, and glutathione (GSH) levels in the cell culture along with inhibition of polyamine biosynthesis. The polysaccharides from soybeans fermented with *P. igniarius* strongly ($p<0.005$) induced QR activity at all concentrations tested. The extract not only induced GST activity in a dose-dependent manner in the concentration range of 0.1–1.0 mg, but significantly induced GSH levels in cultured Hepa 1c1c7 cells with a maximal 1.4-fold increase at 0.1 mg. The polysaccharides from soybeans fermented with *A. cylindracea* were effective in inhibiting polyamine metabolism. These results suggest that polysaccharides from soybeans fermented with *P. igniarius* or *A. cylindracea* have cancer chemopreventive activities in *in vitro* models and, therefore, could be considered as potential agents for cancer chemoprevention.

Key words: Cancer chemoprevention, quinone reductase, glutathione S-transferase, glutathione, polyamine

Cancer is the major cause of death in both men and women, claiming over 6 million lives each year worldwide. Cancer chemoprevention involving preventability, delay, or reversal of the process of carcinogenesis through ingestion of dietary or phamaceutical agents is one of the most direct ways to reduce morbidity and mortality. Induction of phase II drug metabolizing enzymes such as NAD(P)H:quinone oxidoreductase [quinone reductase (QR)] or glutathione S-transferase (GST) is considered as a major mechanism to protect initiation of carcinogenesis, since these enzymes divert ultimate carcinogens from reacting with critical cellular macromolecules [26]. In laboratory animals and cell culture systems, several chemopreventive agents have been identified solely on the basis of their ability to induce phase II enzymes [8, 30]. The induction of reduced glutathione (GSH) was also used to screen potential chemopreventive agents. Glutathione conjugation involves the reaction of electrophiles with the nucleophilic thiol of GSH, which decreases the availability of reactive electrophiles to interact with DNA and possibly initiate the transformation process [11].

Polyamines are essential to achieve optimal cell proliferation in a wide variety of organisms and appear to be important for neoplastic transformation [12]. Phorbol ester promoters such as 12-*O*-tetradecanoylphorbol-13-acetate (TPA) cause accumulation of polyamines in affected tissues [22]. Inhibitors of polyamine biosynthesis are useful or potentially helpful chemotherapeutic agents for treatment of cancer [13]. α-Difluoromethylornithine (DFMO), a specific and irreversible inhibitor of polyamine synthesis, inhibits tumorigenesis induced by a number of different carcinogens [16, 27].

Edible higher basidiomycetes are a nutritionally functional food and a source of physiologically beneficial medicines [18]. Various pharmacological effects have been influenced by mushrooms and mushroom-derived compounds, such as immunological enhancement [6, 7, 9, 24], maintenance of homeostasis, and prevention and improvement of diseases such as cancer, cerebral stroke, and heart diseases [19, 23, 28]. However, some medicinal mushrooms can not be cultivated artificially, and the cost of isolating and purifying compounds in a liquid mass culture is very expensive. Accordingly, it is necessary to develop a fermentation method using solid materials (e.g. soybeans or various cereals) to culture the mycelia of medicinal mushrooms.

We have previously demonstrated the modification of mutagenicities of several mutagens [5, 20], and enhancement of phase II, in addition to antioxidant enzymes in mice [21], by polysaccharides from soybeans, which were fermented with basidiomycetes. In this study, we have investigated the potentials of polysaccharides from soybeans fermented

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with either *P. igniarius* or *A. cylindracea* to induce QR, GST activities, and GSH levels in the cell culture in addition to inhibiting polyamine metabolism, as a preliminary assessment of potential cancer chemopreventive activity.

MATERIALS AND METHODS

Preparation of Inoculum

The strains of *P. igniarius* 26005 and *A. cylindracea* were obtained from the National Agricultural Science and Technology Institute, Korea. Five (5 mm) pieces of the mycelium grown in the solid MYG (malt:yeast:glucose= 1.0:0.4:0.4) media were put into 250 ml of a MYG broth in a 500-ml Erlenmeyer flask. The broth with the *P. igniarius* was grown for 10 days, and that with the *A. cylindracea* was grown for 7 days at 28°C in a shaking incubator (HB201S, Hanback Scientific Co., Seoul, Korea) and then homogenized at 10,000 rpm for 30 s. The homogenates were then used as the inoculum.

Fermentation of Soybeans with *P. igniarius* or *A. cylindracea*

Soybeans (malt soybeans) were soaked with 10 volumes of cold water (18°C), and then water on the surface of the soaked soybeans was removed after 12 h (hydration time). The hydrated soybeans (500 g) were autoclaved. After that, either the *P. igniarius* or *A. cylindracea* homogenate was inoculated into the soybeans, and the two groups were fermented for 15 days at 28°C. Thereafter, both types of fermented soybeans were dried for 48 h at 40°C and stored at −20°C until it was put into use.

Extraction of Polysaccharides

The polysaccharides from the soybeans fermented with *P. igniarius* or *A. cylindracea* were extracted according to the method of Shon *et al.* [18] with the following modifications. The samples were extracted with 10 volumes of boiling water for 3 h at 100°C. After filtration, the filtrates were first precipitated by adding 3 volumes of 95% ethanol overnight at 4°C and then collected by centrifugation (10,000 rpm for 30 min). The precipitates were dissolved in distilled water and centrifuged at 8,000 rpm for 20 min. Finally, the supernatant (polysaccharides) was freeze-dried.

Determination of QR Activity in Cell Culture

The inducer potency for QR was measured in Hepa 1c1c7 murine hepatoma cells grown in 96-well microtiter plates as described earlier [14]. The cells were plated in 96-well plates and grown for 24 h. Each well was refed with a fresh medium containing the test samples. The cells were incubated for an additional 48 h and lysed with 0.8% digitonin and 2 mM of EDTA, pH 7.8. The reaction mixture [0.5 M of Tris-HCl (pH 7.4), bovine serum albumin, 1.5 % Tween-20, 7.5 mM of FAD, 150 mM of glucose-6-phosphate, 50 mM of NADP, yeast glucose-6-phosphate dehydrogenase, 3-(4,5-dimethythiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), and 50 mM of menadione in distilled water] was added to each well. Reaction was arrested after 5 min by adding a solution containing 0.3 mM of dicoumarol in 0.5% DMSO and 5 mM of potassium phosphate, pH 7.4. The plate was then scanned at 595 nm. The protein content was determined by the crystal violet staining of an identical set of test plates. The induction of QR activity was calculated from the ratio of the specific enzyme activity of sample-treated cells in comparison with the solvent control.

GST Activity

The GST activity was measured by using a modified procedure developed by Habig *et al.* [4]. Hepa 1c1c7 murine hepatoma cells were plated in 96-well plates at a density of 1×10^4 cells/well in 200 µl α-minimal essential medium (MEM) supplemented with 10% fetal bovine serum that was grown for 24 h at 37°C in a 5% CO_2 atmosphere. After being incubated for 48 h with the samples, cells were lysed by three repetitive freeze-thaw cycles. For the determination of GST, 100 µl of a freshly prepared reaction mixture containing 2.5 mM of GSH and 1 mM 1-chloro-2,4-dinitrobenzene (CDNB) in 0.1 M of potassium phosphate buffer, pH 6.5, were added to each well. The increase in absorbance at 380 nm is monitored for 3 min with a microtiter plate reader (Molecular Device, Sunnyvale, CA, U.S.A.). The protein content was monitored in duplicate plates by using a bichinchoninic 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 of protein. Data derived from sample-treated cells are compared with the values obtained for the solvent control.

Determination of GSH Levels

The GSH content was assayed by an enzymatic recycling procedure [2] in which it is sequentially oxidized by 5,5'-dithiobis-(2-nitrobenzoic acid) and reduced by an NADPH-generating system (glucose-6-phosphate/glucose-6-phosphate dehydrogenase). The extent of 2-nitro-5-thiobenzoic acid formation was monitored at 405 nm. The GSH content was calculated in comparison with a GSH standard curve. The protein content was determined in duplicate plates prepared and treated as described above, by using a bichinchoninic protein assay kit (Sigma, St. Louis, MO, U.S.A.) with bovine serum albumin as the standard. The GSH levels were expressed as nmol/mg of protein. The induction of GSH levels was calculated from the ratio of the GSH content of the sample-treated cells with the solvent control.

Polyamine Biosynthesis Study

To study the inhibition of polyamine metabolism by the polysaccharides extracts, *Acanthamoeba castellanii* was

grown unagitated at 30°C in a complex broth medium (OGM). Polysaccharides from soybeans fermented with *P. igniarius* or *A. cylindracea* as inhibitors were added during the early exponential phase in 25 cm^2 plastic tissue culture flasks. After incubating for 24–192 h, cell suspensions were prepared and counted with a hemocytometer.

Statistical Evaluation

All data were expressed as mean ± SD (standard deviation). The means were compared by using the Student's *t* test with n=3.

RESULTS AND DISCUSSION

Induction of QR Specific Activity

Induction profiles of the extracts are shown in Fig. 1. The polysaccharides from soybeans fermented with *P. igniarius* significantly induced QR activity with a maximum of 3.1-fold induction at the concentration of 0.1 mg. The polysaccharides from soybeans also induced QR enzyme activity considerably in cultured Hepa 1c1c7 cells. However, the extract from soybeans fermented with *A. cylindracea* moderately increased the QR activity (Fig. 1). Our results clearly showed that the polysaccharides from soybeans fermented with *P. igniarius* or *A. cylindracea* were able to induce QR activity. Although the mode of action of some basidiomycetes on QR activity is not clearly understood, it is possible that the increase in QR activity that was induced by polysaccharides from soybeans fermented with *P. igniarius* or *A. cylindracea* was due to the combined effects of many compounds of these extracts.

The QR enzyme induction pattern shows characteristic consequences following the administration of many types of compounds (e.g., phenolic antioxidants, isothiocyanates, 1,2-dithiole-3-thiones, thiocarbamates, coumarins) that protect animals against the carcinogenic and other toxic effects of a wide variety of chemical agents [29].

GST Activity

The effects of the polysaccharides extracts on the induction of GST activity are shown in Fig. 2. GST activity was slightly increased with the polysaccharides from soybeans fermented with *A. cylindracea*. The extract from soybeans fermented with *P. igniarius* induced GST activity in a dose-dependent manner in the concentration range of 0.1–1.0 mg, with a maximum of 1.5-fold induction at the highest concentration tested. The result revealed that polysaccharides from soybeans fermented with *P. igniarius* or *A. cylindracea* were also associated with induction of the phase II detoxification enzyme, glutathione S-transferase. GST catalyzes the conjugation of many electrophilic compounds with glutathione. The enzyme also performs a number of other functions in cellular metabolism, such as reduction of organic hydroperoxides and steroid isomerization. Since electrophiles may bind to nucleophilic compounds such as proteins and DNA, conjugation with glutathione protects the cell against these reactive compounds. In addition, GST protects the cell against organic hydroperoxide because of their peroxidase activity. Consequently, induction

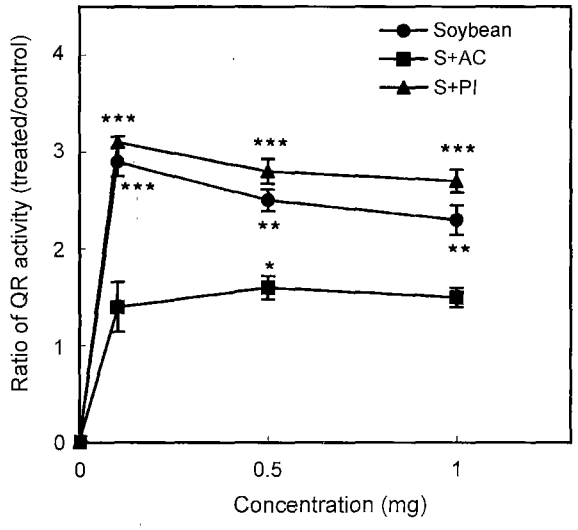

Fig. 1. Effect of polysaccharides from soybeans and soybeans fermented with *A. cylindracea* (S+AC) or *P. igniarius* (S+PI) on induction of quinone reductase (QR) activity in mouse hepatoma Hepa 1c1c7 cells.
Each point represents mean±SD (standard deviation) of three separate experiments. The mean is statistically significant according to a Student's *t* test (*p<0.05, **p<0.01, ***p<0.005).

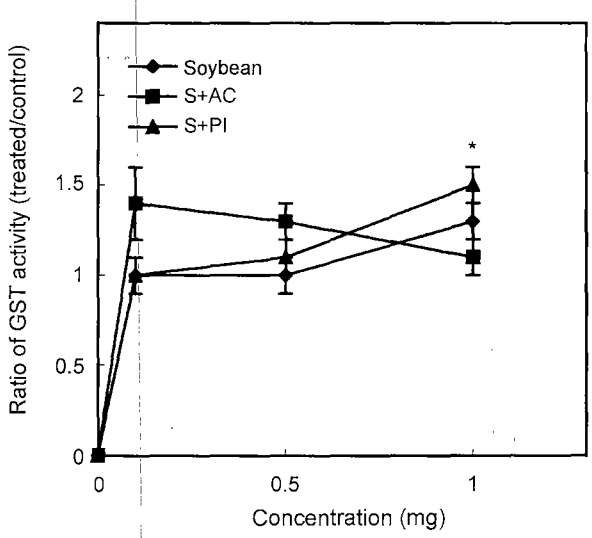

Fig. 2. Effect of polysaccharides from soybeans and soybeans fermented with *A. cylindracea* (S+AC) or *P. igniarius* (S+PI) on induction of glutathione S-transferase (GST) activity.
Each point represents mean±SD (standard deviation) of three separate experiments. The mean is statistically significant according to a Student's *t* test (*p<0.05).

of GST is mostly assumed to result in a decreased cancer risk [25]. Highly reactive epoxides of numerous compounds, including benzo[a]pyrene, are substrates for human GST. The dithiolethione, oltipraz, is a potent inducer of GST and inhibits carcinogen-induced tumorigenesis in a number of animal models [15, 17].

A number of previous studies have suggested that induction of phase II detoxification enzymes, such as QR and GST is a relevant mechanism for cancer chemoprevention [1, 3, 30]. These inducible enzymes facilitate the metabolic detoxification of xenobiotics in mammals and can achieve chemopreventive activity by modification of carcinogen metabolism through increased carcinogen excretion and decreased carcinogen-DNA interaction.

GSH Levels

In addition to the induction of phase II enzymes, the polysaccharides from soybeans fermented with *P. igniarius* or *A. cylindracea* slightly induced glutathione levels in cultured Hepa 1c1c7 cells (Fig. 3). When large doses of GSH were given to the rats bearing aflatoxin-induced liver tumors, the result was a substantial reduction of these tumors. Furthermore, butylated hydroxyanisole, a food additive, has been found to inhibit chemical carcinogenesis by increasing levels of GSH [10].

In this study, QR and GST activities and GSH levels were elevated by polysaccharides from the soybeans fermented with *P. igniarius* or *A. cylindracea*. These extracts were found to induce drug-metabolizing enzymes in a cell culture, and chemopreventive activity was attributed to the increased detoxification of xenobiotics and carcinogens, for the most part.

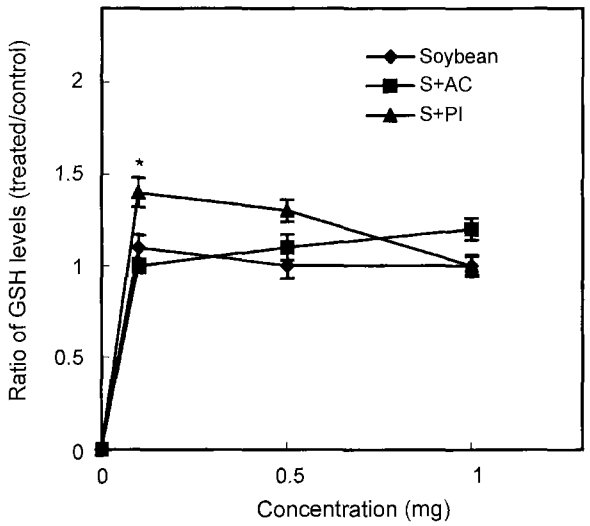

Fig. 3. Effect of polysaccharides from soybeans and soybeans fermented with *A. cylindracea* (S+AC) or *P. igniarius* (S+PI) on induction of glutathione (GSH) levels in mouse hepatoma Hepa 1c1c7 cells.
Each point represents mean±SD (standard deviation) of three separate experiments. The mean is statistically significant according to a Student's *t* test (*p<0.05).

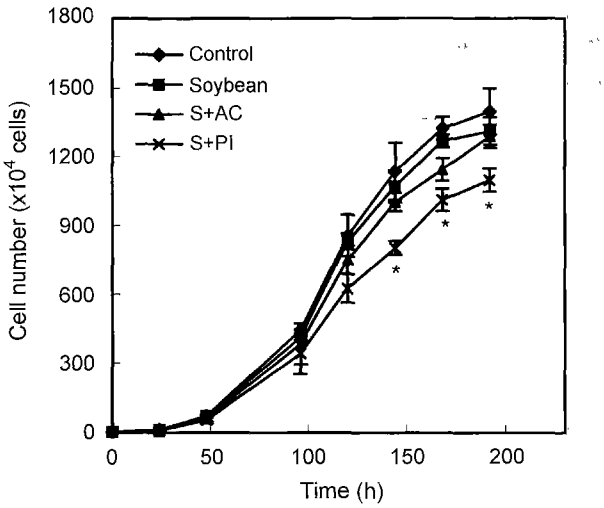

Fig. 4. Inhibition of the growth of the *Acanthamoeba castellanii* by polysaccharides from soybeans and soybeans fermented with *A. cylindracea* (S+AC) or *P. igniarius* (S+PI) at the concentration of 20 mg. *A. castellanii* was incubated with samples for each time period.
Each point represents mean±SD (standard deviation) of three separate experiments. The mean is statistically significant according to a Student's *t* test (*p<0.05).

Inhibition of Polyamine Metabolism

Growth of *A. castellanii* was inhibited by the extracts from soybeans fermented with *P. igniarius* or *A. cylindracea* at

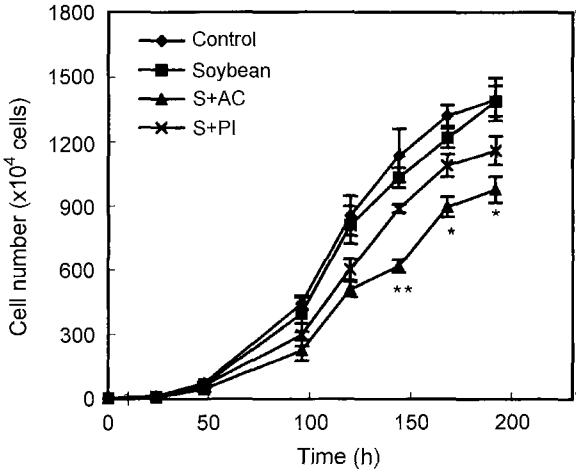

Fig. 5. Inhibition of the growth of the *Acanthamoeba castellanii* by polysaccharides from soybeans and soybeans fermented with *A. cylindracea* (S+AC) or *P. igniarius* (S+PI) at the concentration of 40 mg. *A. castellanii* was incubated with samples for each time period.
Each point represents mean±SD (standard deviation) of three separate experiments. The mean is statistically significant according to a Student's *t* test (*p<0.05, **p<0.01).

20 mg (Fig. 4) and 40 mg (Fig. 5). Concentrations higher than those described lysed cells. The arrest of multiplication by 20 mg of the polysaccharides from soybeans fermented with *P. igniarius* ($p<0.05$, Fig. 4) and 40 mg of the polysaccharides from soybeans fermented with *A. cylindracea* ($p<0.05$ and $p<0.01$, Fig. 5) was significant. The polyamine content of cells was correlated to their proliferative and neoplastic capabilities. As tumor formation can be prevented by the agents that block polyamine biosynthesis, such as retinoids, α-difluoromethylornithine, or inhibitors of arachidonic acid metabolism including indomethacin [13], inhibition of polyamine metabolism was shown to be a promising tool for screening inhibitors of tumorigenesis.

In conclusion, the chemopreventive potentials of the polysaccharides from soybeans fermented with *P. igniarius* or *A. cylindracea* were evaluated by measuring the induction of phase II metabolizing enzyme and glutathione levels along with the inhibition of polyamine biosynthesis. The polysaccharides from soybeans fermented with *P. igniarius* were found to be the most effective to induce QR and GST activities and GSH levels. The polysaccharides from soybeans fermented with *A. cylindracea* were effective in the inhibition of polyamine metabolism. Such results suggest that there is a difference in potential chemopreventive activity between polysaccharides from soybeans fermented with *P. igniarius* and polysaccharides from soybeans fermented with *A. cylindracea*. Since the implication of more than one compound in the chemopreventive activity of the extracts may not be ruled out, the isolation of these may be a prerequisite to unravelling the probable mode of action. However, these data provide useful information needed for further development of extracts from soybeans fermented with basidiomycetes as chemoprevention agents, in animal studies, and subsequently, in human clinical trails.

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