Induction of Anticarcinogenic Enzymes of Waxy Brown Rice Cultured with *Phellinus igniarius* 26005

Ki-Bum Park, Hyo-Cheol Ha, So-Yeun Kim, Hyo-Jeong Kim and Jae-Sung Lee*

Department of Food Science and Technology, Yeungnam University, Kyungsan 712-749, Korea

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The induction of NAD(P)H: quinone oxidoreductase (QR), glutathione S-transferase (GST), and glutathione (GSH) levels in hepatic cells (murine hepatoma) by waxy brown rice cultured with *Phellinus igniarius* to induce anticarcinogenic enzymes were measured. In addition, the inhibition of polyamines metabolism was tested with the growth of *Acanthamoeba castellanii*. The result shows that QR, GST activities, and GSH levels of experimental animals were increased much more by feeding the methanol extract of waxy brown rice cultured with *Phellinus igniarius* than those of the rats received the ethanol of uncultured brown rice. The growth of *A. castellanii* was inhibited mostly at 40 mg/3 ml concentration of methanol extract of waxy brown rice cultured with *P. igniarius*. The results suggested that waxy brown rice cultured with *P. igniarius* possess chemopreventive activity by inducing anticarcinogenic enzymes and inhibiting polyamine metabolism.

KEYWORDS: Chemoprevention, Glutathione, Glutathione S-transferase, NAD(P)H *Phellinus igniarius*, Quinone oxidoreductase

The cause of cancer is diverse, but 80~90% of cancer is related to environmental factors. Among them, it is reported, 30~60% was directly or indirectly connected with a diet (Doll *et al*., 1981). Recently, a new approach to protect cells from carcinogens, called chemoprevention, has been introduced. Chemoprevention is recognized as the most realistic way of primary prevention of cancer, and one of the promising alternative strategy, blocking or preventing the development of invasive cancers caused by the use of natural or synthetic compounds (Ferguson, 1999; Wattenberg, 1985). Initial reactions of several tumors occur by metabolite of electrophilic carcinogens or active oxygen injuring DNA (Preter et al., 1993). However, mammalian cells have exquisite mechanisms to protect those damage, namely, detoxification enzymes prevent the development of cancers by eliminating electrophilic carcinogens out of body after detoxifying them. It has been suggested that phase II detoxification enzymes such as UDP-glucuronosyltransferases, NAD(P)H: quinone oxidoreductase (QR), glutathione S-transferases (GST), in addition to the increasing the glutathione (GSH) levels, play a role in the cellular detoxification of carcinogens (De Long *et al*., 1986; Talalay *et al*., 1995). Additionally, polyamines are related to proliferation of tumor cells, and abnormally biosynthesized in carcinogenesis, and therefore, inhibition of polyamine metabolism is useful for cancer prevention (Bull *et al*., 1984).

Many mushrooms belong to basidiomycetes forming fruit body, and have been used for foods or medicinal use from ancient times. In addition, mushrooms are low calorie foods, but rich in protein, chitin, vitamins, and minerals (Manzi *et al*., 1999; 2001). The advantage of mushrooms is that they have no or low harmful side effects, unlike other medicines (Lee, 1996). Numerous researches have been carried out investigations for antitumor activities, antimutagenic activities or other functions of mushrooms (Mizuno *et al*., 1992; Shon and Nam, 2001). However, the mechanism of cancer chemopreventive effects of basidiomycetes are not well known.

In this study, chemopreventive potentials centering around phase II enzymes with determination of polyamines metabolism inhibition were investigated to proclaim the functional activities of waxy brown rice cultured with *Phellinus igniarius* 26005 which already have been developed in Functional Food Lab. of Yeungnam university.

Materials and Methods

**Strain.** *Phellinus igniarius* 26005 (here in after referred as PI), obtained from Dept. of Applied Microbiology in National Institute of Agriculture Science and Technology.

**Preparation for sample.** Waxy brown rice was soaked with 10-fold volume of water (18°C) for 12~14 hours, and drained by keeping by itself for 2~3 hours. The washed waxy brown rice was packed in plastic bag, and steamed at 121°C for 2 hours. After cooling at room temperature, waxy brown rice was inoculated with the homogenized mycelium of PI, and cultured for 14 days at 28±1°C. The waxy brown rice cultured with PI was extracted by 5-fold volume of methanol or water for 24 hours. The extracts were filtered, concentrated, and then freeze dried. The freeze dried samples were stored at −80°C and used in this experiment.
Determination of QR (Quinone reductase) activity

**QR assay:** Intracellular QR activity was measured by the method of Prochaska et al. (1988) using Hepa1c1c7 cells. Hepa1c1c7 cells were plated in 96 well microtiter plates at a density of 1×10⁴ cells/well in 200 µl of MEM supplemented with 10% FBS. The cells were incubated for 24 hours in a humidified incubator with 5% CO₂ at 37°C. The medium was decanted, and each well was refed with 200 µl of medium containing the extracts of waxy brown rice cultured with PI. The cells were then cultured for additional 48 hours. The culture media were aspirated and the cells in each well were lysed by 3 repetitive freeze-thaw cycles. The 200 µl of reaction mixture [0.5 M Tris-HCl (pH 7.4), bovine serum albumin, 1.5% Tween-20, 7.5 mM FAD, 150 mM glucose-6-phosphate dehydrogenase, 50 mM NADP, 3 mg/10 ml of 3-[4,5-dimethyl-azol-2-yl]-2,5-diphenyltetrazolium bromide (MTT), and 50 mM menadione in distilled water] was added to each well, and reacted for 5 min. To arrest the reaction, a solution containing 0.3 mM diconarol in 0.5% DMSO and 5 mM potassium phosphate (pH 7.4) was added, and the absorptions were measured at 570 nm.

**Cytotoxicity assay (crystal violet staining):** Hepa1c1c7 cells were plated in 96 well microtiter plates, and cultured for 24 hours in the same method as QR assay. The plates were treated with waxy brown rice cultured with PI, and then incubated for additional 48 hours. The media were decanted, and the plates were submerged in a 0.2% crystal violet solution dissolved in 50% ethanol to dissolve. The absorptions were measured at 570 nm.

**Calculation of QR activity:** QR activity was determined with the MTT reduction and crystal violet absorbances. QR activity induction was calculated by the ratio of QR activity of the sample group against QR activity of control group. The formula for calculation of QR activity is as follow:

\[
\text{Specific activity} = \frac{\text{absorbance change of MTT/min}}{\text{absorbance of crystal violet}} \times 3345 \text{ nmol/mg}
\]

**Determination of GST (Glutathione S-transferase) activity:** Intracellular GST activity was tested according to Habig et al. (1974) by measuring the enzymatic coupling of CDNB (1-chloro-2,3-dinitro benzene) to GSH. Hepa1c1c7 cells were plated in 96 well microtiter plates, and cultured for 24 hours in the same method as QR assay. The plates were treated with sample extracts, and then incubated for additional 48 hours. The media were decanted, and the plates were rinsed 3 times with PBS. The cells were lysed by 3 repetitive freeze-thaw cycles, and 100 µl of reaction mixture [1 mM CDNB and 2.5 mM GSH in potassium phosphate buffer (pH 6.5)] was added to each well. The plates were shaken for 1 min on a microplate shaker, and reacted for 60 min at room temperature under slow continuous shaking. The absorptions were measured with a microplate reader at 405 nm. The protein contents were monitored in duplicate plates by a bicinchoninic protein assay kit using bovine serum albumin (BSA) as a standard solution. GST activity was expressed in arbitrary units. The units were calculated as follows:

\[
\text{Specific activity} = \frac{\text{OD of sample (mean of 3)} - \text{OD of buffer}}{\text{protein content (mg)}}
\]

GST activity of cells treated with sample extract was compared with the activity of control cells treated with medium only.

**Determination of GSH (Glutathione) levels.** To determine the total intracellular GSH levels, the method of Griffith (1980) was appropriately modified to perform at 96 well microtiter plates. Hepa1c1c7 cells were plated in 96 well microtiter plates, and cultured for 24 hours in the same method as QR assay. The plates were treated with sample extracts, and then incubated for additional 48 hours. The media were decanted, and the plates were rinsed 3 times with PBS. The cells were lysed by 3 repetitive freeze-thaw cycles, and 40 µl of stock buffer [125 mM sodium phosphate, 6.3 mM ethylenediamine tetracetic acid (Na-EDTA), pH 7.4] and 170 µl reaction mixture [20 µl of 6 mM 5,5′-dithiobis(2-nitro-benzoic acid)(DTNB) in stock buffer, 10 µl of glutathione reductase solution (50 unit in 10 ml of stock buffer), 140 µl of NADPH-generating system, 330 µl of 150 mM glucose-6-phosphate, 30 µl of 50 mM NADP, 100 unit glucose-6-phosphate dehydrogenase and distilled water to a final volume of 50 ml] was added to lysed each well. The plates were shaken for 5 min at room temperature before measuring at 450 nm. GSH levels were determined according to the GSH standard curve, and expressed by the ratio of GSH levels of the group treated with sample extracts against those of the group treated with the culture medium. The protein contents were monitored in duplicate plates using a bicinchoninic protein assay kit with bovine serum albumin as the standard.

**Determination of polyamine metabolism.** *Acanthamoeba castellanii* strain used in this study was obtained from American Type Culture Collection (Maryland, USA). Cells were grown in T-75 flasks with 10 ml of Neff’s growth medium designated OGM (Kim et al., 1987) at 30°C under anaerobic condition in the dark, and subcultured twice a week. The cells were inoculated at a density of 1×10⁷/2.5 ml of media in T-25 flasks. After incubation...
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for 24 hours at 30°C, the sample extracts were added as a solution with 0.5 ml of media. The cells were incubated for 168 hours, and were counted every 24 hours (Byers et al., 1980).

Statistical evaluation. The data were exhibited by average ± SD (standard deviation), and analysed using SPSS program.

Results and Discussion

Induction of QR enzyme. QR is primarily a cytosolic flavoprotein that catalyzes the reduction of a wide variety of quinones and quinoneimines (Prochaska et al., 1987, 1988). The quinones are known to be cytotoxic due to their readiness to undergo reduction-oxidation cycles. They are widely distributed in nature, and human is extensively exposed to them. With other phase II enzymes such as glutathione S-transferase, UDP-glucuronosyltransferase, QR protects cells against carcinogens as well as prevents mutation, by reducing quinones or quinoneimines. QR is observed the most highly in liver, and relatively high in kidney and brain (Lee et al., 1999).

QR induction in cultured hepa1c1c7 cells treated with extracts of waxy brown rice cultured with PI was measured to assess chemopreventive potentials (Fig. 1). Waxy brown rice cultured with PI induced QR activity more than 3 times that of control, and specially, methanol extract reached 3.5 times in comparison with control at concentration of 5 mg/ml. QR inductive activities increased in proportion to the concentration of extracts, i.e. dosage dependant.

Shon et al. (2001) reported that polysaccharides isolated from the cultured broth, and mycelia of Phellinus igniarius were effective inducer of phase II detoxification enzyme. Furthermore, Hong et al. (1997) reported that methanol extract from roasted perilla induced QR enzymes. Benson et al. (1980) found the antioxidant actions of phenolic antioxidants can be ascribed to the induction of QR. This study suggest that waxy brown rice cultured with PI is a good QR inducer, and might contribute in blocking intracellular toxicity of carcinogens.

Induction of GST enzyme. GST inductive activity was investigated to measure chemopreventive potentials of waxy brown rice cultured with PI using hepa1c1c7 cells. The results were shown in Fig. 2. The extracts of uncultured waxy brown rice did not show substantial increase in GST activity at concentration of 0.1~5 mg/ml. The highest activity was shown by methanol extract of waxy brown rice cultured with PI by increasing GST activity up to 79% at the same concentration. While the concentration of water extracts has not much to do with the GST activity, the GST activity was increased by methanol extracts concentration dependently up to 2 mg/ml. Kim et al. (1999) reported that the extract from Ganoderma lucidum increased 20~30% of GST activity.

Carcinogens are detoxified via activation or inactivation of enzymes in liver. The GSTs are a family of enzymes which assist the excreting of carcinogens after making them soluble via conjugation with glutathione (GSH) (Bora et al., 1989). Until now, seven soluble GST gene subfamilies (alpha, mu, pi, sigma, kappa, theta and zeta) have been identified in mammals (Mennervik et al., 1992). In phase II detoxication process, the GSTs catalyze the conversion of reactive intermediates through cytochrome P450 (phase I detoxication), and form a protective system against carcinogens by inactivation of electrophilic compounds. Thus, the GSTs, one of phase II enzymes, are used as a biomarker to investigate chemopreventive effects of agents (Williamson et al., 1997).

This study show that waxy brown rice cultured with PI...
may contribute to hinder cancerization by excreting electrophilic carcinogens in the stage of cancer development, via inducing GST activity.

**GSH levels.** Glutathione, consisted of glutamic acid, cystein and glycine, is the thiol compound present in the highest concentration in mammalian cells. Glutathione plays an important role as a substrate for GST, and excretes after detoxification by reacting directly with metabolite of electrophilic carcinogens (Dringen, 2000; Lesley et al., 1999).

In this study, intracellular GSH levels were measured to demonstrate chemopreventive potentials of waxy brown rice cultured with PI using hepa1c1c7 cells (Fig. 3). Comparing with control, the treatment group with methanol extract of waxy brown rice showed 17~47% increase, while waxy brown rice cultured with PI increased GSH levels by 20~70%. There was a tendency that GSH levels were increased concentration dependently up to concentration of 2 mg/ml, but it dropped obviously at concentration of 5 mg/ml.

The low level of GSH in a body can cause various kinds of diseases in liver, and depletion of GSH causes cancer or damage of cells due to production of strong toxic metabolites. Preserving normal GSH levels or prohibiting drastic GSH shifts would maintain the integrity of normal signaling and act to stabilize the cell from detrimental redox fluctuations (Michell et al., 1976).

Accordingly, waxy brown rice cultured with PI did not induce noticeable GSH levels compared with waxy brown rice, but it may protect cells against carcinogens by activating detoxication enzymes.

**Polyamine metabolism inhibition.** The growth of *Acanthamoeba castellanii* was measured to investigate the inhibition of polyamine metabolism. *A. castellanii* was inoculated to OGM media and after 24 hours of incubation the flask was treated with the extracts from waxy brown rice cultured with PI. The cell number at that point was $9.9 \times 10^7$. The cell count after 48 hours of incubation reached to $42.9 \times 10^4$ in control, $30.4 \times 10^4$ in the treatment group with 20 mg water extract of waxy brown rice and $25.4 \times 10^4$ in the treatment group with 20 mg water extract of waxy brown rice cultured with PI, respectively. After 168 hours of incubation, the cell count of control was observed to be $2.2 \times 10^7$ while water extract of waxy brown rice inhibited the growth down to $1.8 \times 10^7$. Water extract of waxy brown rice cultured with PI exhibited the highest inhibitory effect down to $1.6 \times 10^7$ of cell number (Fig. 4).

In groups treated with 40 mg of water extracts, the highest inhibitory effect was shown by the group treated with water extract of waxy brown rice cultured with PI, down to $1.3 \times 10^7$ of cell number after 168 hours of incubation (Fig. 5).

A greater inhibition of *A. castellanii* proliferation was observed in the group treated with methanol extracts. In the groups treated with 20 mg of methanol extracts, the cell count after 48 hours of incubation reached to $28.6 \times 10^4$ in the treatment group with methanol extract of waxy brown rice and $23.2 \times 10^4$ in the treatment group with methanol extract of waxy brown rice cultured with PI, respectively. After 168 hours of incubation, the cell count of control was observed to be $2.1 \times 10^7$ while methanol extract of waxy brown rice cultured with PI inhibited the growth down to $1.4 \times 10^7$ of cell number (Fig. 6).

In the groups treated with 40 mg of methanol extracts, the highest inhibitory effect was shown by the group...
treated with methanol extract of waxy brown rice culture with PI, down to $1.3 \times 10^7$ of cell number (Fig. 7).

Polyamines play an essential role in cell proliferation and differentiation, and cellular polyamine levels are strictly feedback-regulated to avoid cell injury or death by excessive cellular polyamine accumulation. Therefore, inhibition of polyamine biosynthesis is important mechanism to screen active materials having functions to prevent cancer promotion and proliferation of tumor in promotion phase (Pegg et al., 1982; Tabor et al., 1984). Kim (2000) studied the inhibition of polyamine metabolism with soybean cultured with PI, and reported soybean cultured with PI had the highest inhibitory effect. The pathway of polyamine biosynthesis is controlled by ornithine decarboxylase (ODC), and ODC catalyses the synthesis of putrescine from ornithine in mammalian cells. The molecular weight of ODC is 52,000, and is induced by various stimuli such as hormones, growth factors, tumor promoters, etc. Difluoromethyl ornithine (DFMO), well known as an inhibitor of polyamine metabolism, hinders the activity of ODC and blocks cancerization by inhibiting the growth of tumor cells (Kingsnorth et al., 1983; Rozhin et al., 1984).

This result suggested that waxy brown rice cultured with PI may block proliferation of tumor cells due to its inhibitory effect for polyamine metabolism also.

References


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