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Transcriptional Activation of Cu/Zn Superoxide Dismutase and Catalase Genes by Panaxadiol Ginsenosides Extracted from *Panax ginseng*

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Superoxide dismutase (SOD) converts superoxide radical to H_2O_2, which is in turn broken down to water and oxygen by catalase. Thus, SOD and catalase constitute the first coordinated unit of defence against reactive oxygen species. A wide variety of chemical and environmental factors are known to induce these antioxidant enzymes. Here, we examined the effect of ginseng saponins on the induction of SOD and catalase gene expression. To explore this possibility, the upstream regulatory promoter region of Cu/Zn superoxide dismutase (SOD1) and catalase genes were linked to the chloramphenicol acetyltransferase (CAT) structural gene and introduced into human hepatoma HepG2 cells. Total saponin and panaxatriol did not activate the transcription of SOD1 and catalase genes but panaxadiol increased the transcription of these genes about 2–3 fold. Among the panaxadiol ginsenosides, the Rb_2 subfraction appeared to be a major inducer of SOD1 and catalase genes. The specificity of the Rb_2 effect was further confirmed by time course- and dose-dependent induction experiments. These results suggest that the panaxadiol fraction and its ginsenosides could induce the antioxidant enzymes which are important for maintaining cell viability by lowering the level of oxygen radical generated from intracellular metabolism.

Keywords: Panax ginseng; panaxadiol ginsenosides; induction; SOD1, catalase genes.

INTRODUCTION

All aerobic organisms produce reactive oxygen species, such as superoxide radicals and hydrogen peroxide, both spontaneously and as a result of enzymatic activity of oxidative enzymes (Malmstrom, 1982). Cu/Zn superoxide dismutase (SOD1) is a protective enzyme responsible for maintaining a low level of superoxide radicals within the cell by converting superoxide radicals to oxygen and hydrogen peroxide, which is in turn changed to oxygen and water by catalase. It has also been reported that SOD1 could prevent oncogenesis and tumour promotion, reduce the cytotoxic and cardiotoxic effects of anticancer drugs, and protect against reperfusion damage of ischaemic tissue (McCord *et al.*, 1971; Fridovich, 1983). Another line of study proposed that the damage stems from its ability to interact with H_2O_2 to generate singlet oxygen and hydroxyl radicals, which are extremely active and highly cytotoxic forms of oxygen (Badwey and Karnovsky, 1980). The activity of SOD1 is closely related to catalase, which is a tetrameric hemeprotein found in peroxisomes and which catalyses the decomposition of hydrogen peroxide to oxygen and water. Catalase is found in virtually all aerobic cells and is partly responsible for protecting cells against the toxic effects of hydrogen peroxide. Overproduction of SOD1 alone or catalase alone had only minor incremental effects on the average life span of *Drosophila* (Orr and Sohal, 1994). It is therefore of importance to explore the inducer which triggers the expression of antioxidant enzymes.

Panax ginseng C. A. Mayer (Araliaceae) is one of the most popular natural tonics and has been shown to possess various biological activities such as a protein anabolic effect, antitumour activities and an inhibitory effect of tumour angiogenesis and metastasis (Sato *et al.*, 1994). Also, ginseng has been used for treatment of heart failure and to protect tissues from damage when an organism is in stress (Wagner and Liu, 1987). Moreover, ginseng has the advantage that it is free from harmful side effects. Saponin, the active fraction of ginseng, is classified largely into two groups, panaxadiol (PD) and panaxatriol (PT) saponins, which differ in sugar moiety at the position of carbon-3,-6 and -20 (Sanada *et al.*, 1974; Shoji, 1974).

Recently, we reported that ginsenoside Rb_2 activated SOD1 transcription through transcription factor AP2 binding sites (Kim *et al.*, 1996). Here, we examined the total saponin, panaxadiol, panaxatriol and each major ginsenoside for induction of the SOD1 and catalase genes. Panaxadiol appeared to be a good inducer for both genes and its ginsenoside Rb_2 was the best. These results suggest that the panaxadiol fraction, which is relatively easier to obtain than its subfractions, would be a useful candidate for the induction of antioxidant enzymes against reactive oxygen species.

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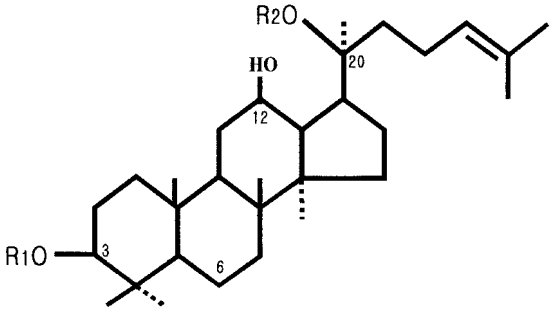

Figure 1. Basic structure of 20(s)-protopanaxadiol. Panaxadiol constitutes the common R_1 site: -glc(2 → 1)glc. The R_2 site in each ginsenoside differs as follows: ginsenoside Rb_1, glc(6)-glc; ginsenoside Rb_2, glc(6)-ara(pyr); ginsenoside Rc, glc(6)-ara(fur); ginsenoside Rd, glc.

MATERIALS AND METHODS

Plant materials and plasmid construction. Total saponin, panaxadiol, panaxatriol and ginsenoside-Rb_1, -Rb_2, -Rc and -Rd were isolated from 6-year-old red ginseng by thin layer chromatography and high performance liquid chromatography (Sanada *et al.*, 1974; Shoji, 1974) and supplied by Korea Ginseng and Tobacco Research Institute. Plasmid SODCAT, which contains the promoter region of SOD1 gene, was constructed as follows: the 1.7-kilobase pair *Bam*HI/*Sma*I fragment (nucleotides −1633 to +85) of the promoter region from the rat SOD1 gene (Kim *et al.*, 1993) was inserted into pBLCAT2 (Luckow and Schütz, 1987). Plasmid pBluCLCAT, which contains the promoter region of the catalase gene, was prepared by polymerase chain reaction using synthetic oligonucleotide primers (5′-CGCGGATCCATAATACTTACATTAGCGTATGG-CA-3′, 5′-AAACTGCAGAA-CACTGCACTGCACTG-CAGGAGGCCTCGGCT-3′), which amplified the genomic sequence from −554 to −27 of the promoter. The cloned fragment was inserted into the *Bam*H1 and *Pst*1 sites of the multiple cloning site of pBluCAT, which was created by inserting the CAT gene of pBluscriptSK. The sequence of the cloned catalase upstream region was verified by DNA sequence analysis.

Cell culture, transfection, and treatment of ginseng saponins. Human HepG2 hepatoma cells were grown in Dulbecco's modified Eagle's medium (DMEM) 10% fetal calf serum/ penicillin G sodium at 100 units per mL/ streptomycin sulphate at 100 μg/mL amphotericin B at 250 ng/mL. Cells were seeded into 60 mm plastic culture dishes (30%–50% confluence) for 24 h prior to transfection. An equal amount (3.0 pmol) of SODCAT or pBluCLCAT construct was transfected to the cells by the calcium phosphate DNA coprecipitation method (Chen and Okayama, 1987). A 5 μg sample of pRSV*β*-gal plasmid (Edlund *et al.*, 1985) was also introduced in all experiments to correct for variations of transfection efficiency. Ginseng saponins were added to culture medium at 36 h after transfection and the cells were maintained for an additional 22 h. To determine the maximum induction time of Rb_2, the growth medium was removed and the Rb_2 was added to the cells at 50 μM (SODCAT) or 100 μM (pBluCLCAT) in phosphate-buffered saline (PBS). After treatment for 30 min at 37 °C, the growth medium was added back to the cells and incubation was continued for proper intervals as indicated.

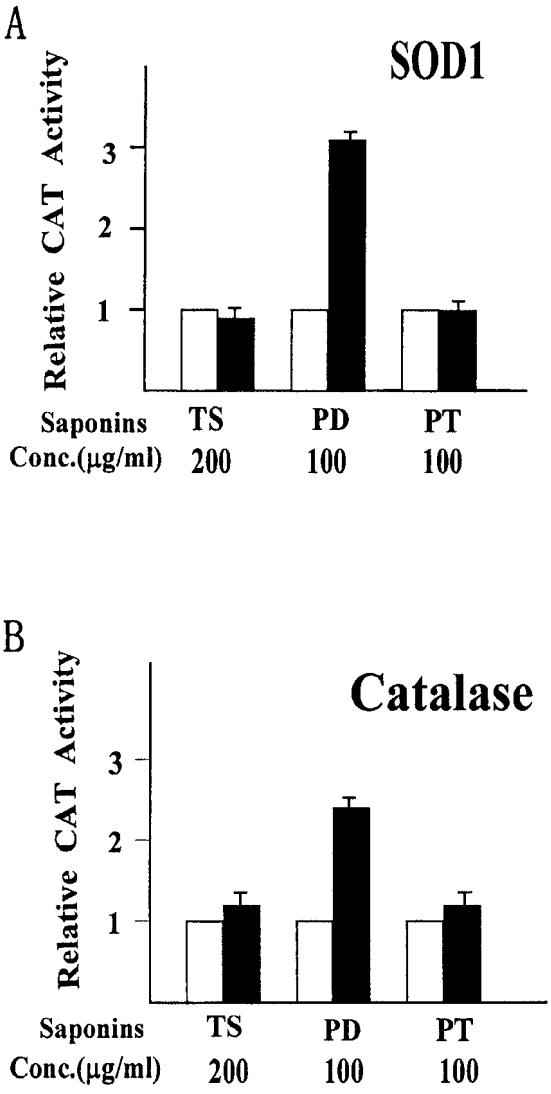

Figure 2. Effect of ginseng saponins on the induction of SOD1 and catalase genes. Total saponin (TS), panaxadiol (PD) and panaxatriol (PT) were treated as indicated in Materials and Methods. CAT activity was measured 70 h after reporter plasmid transfection. Solid bar indicates the CAT value with saponins and open bar without saponins. The results of CAT assay are representatives of the mean ±SD of three independent experiments.

β-galactosidase and CAT assays

The CAT assay was performed as described (Gorman *et al.*, 1982). The transfected cells were washed twice with PBS and harvested. The pelleted cells were resuspended in 100 μL of 0.25 M Tris-Cl (pH 7.9) and lysed by three cycles of freezing and thawing. After removing cell debris by centrifugation, cell extracts were first assayed for *β*-galactosidase activity (Sambrook *et al.*, 1989). Equal quantities of proteins were assayed for CAT activity on the basis of *β*-galactosidase activity. Extracts were incubated with 0.025 μCi of [^{14}C] chloramphenicol/ 0.25M Tris-Cl pH 7.6/ 0.4 mM acetyl coenzyme A for 1 h at 37 °C. The reaction was terminated by adding ethylacetate. The organic layer was analysed by TLC with chloroform/methanol (95:5). After autoradiography,

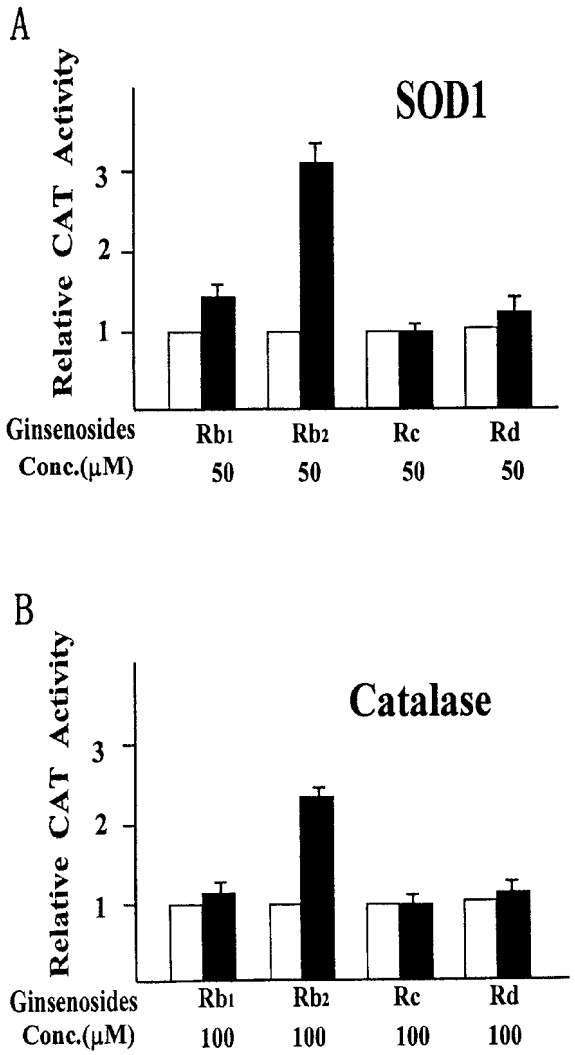

Figure 3. Effect of major ginsenosides of PD on the induction of SOD1 and catalase genes. After transfection for 36 h, ginsenoside -Rb_1, -Rb_2, -Rc and -Rd were added for 22 h before CAT assay. Solid bar indicates the CAT value with saponins and open bar without saponins. The results of CAT assay are representatives of the mean ±SD of three independent experiments.

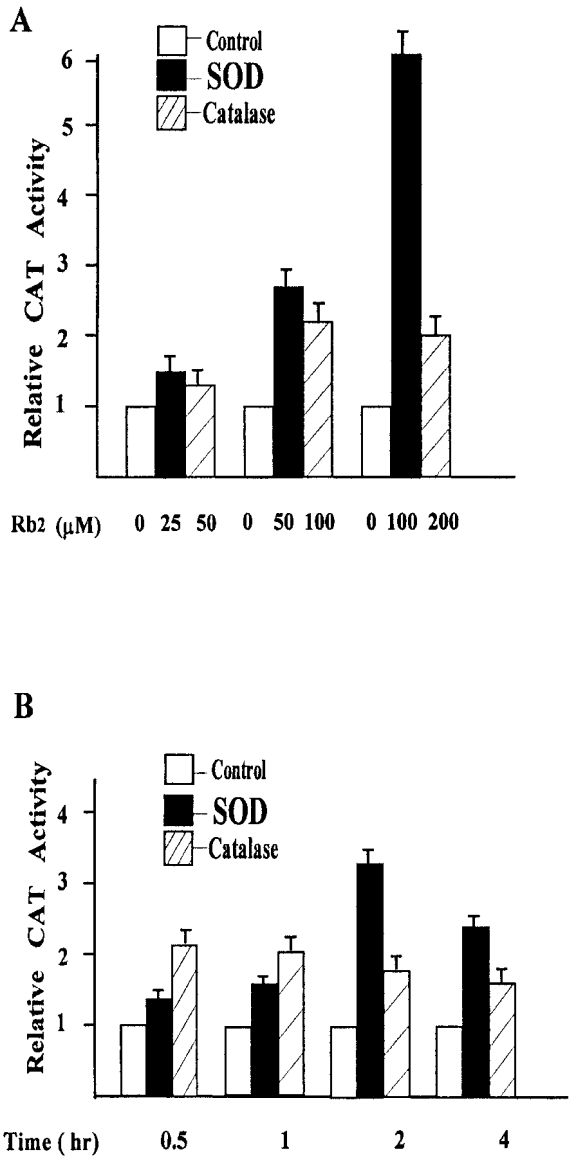

Figure 4. Effects of the concentration and treatment time of Rb_2 on expression of the SOD1 and catalase genes. (A) Treatment of the cell with increasing amounts of Rb_2. (B) Time course induction profiles. To determine the maximum induction time for Rb_2, the growth medium was removed and Rb_2 added to the cells at 50 μM (SODCAT) or 100 μM (pBluCLCAT) in phosphate-buffered saline (PBS). Treatments were maintained for 30 min at 37 °C, and the Rb_2 was removed and new growth medium was added for the continuing incubation as indicated. The results of CAT assay are representatives of the mean ±SD of three independent experiments.

both acetylated and unacetylated forms of [^{14}C] chloramphenicol were scraped from the plate and the conversion of chloramphenicol to the acetylated form was calculated by measuring radioactivities. The relative CAT activities were calculated from the percent conversion.

RESULTS AND DISCUSSION

The formation of oxygen radicals and cytotoxic oxygen metabolites probably play a key role in various types of tissue degeneration and pathology such as aging, cancer and retinal degeneration (Brown, 1995). In order to overcome the effect of free radicals and to reduce the damage by oxidants, a variety of pharmacological antioxidants such as glutathione, celluloplasmin and transferrin have been examined (Gutteridge, 1986). In this study, we demonstrated the effect of ginseng saponin, which is known to possess non-harmful side effects, on the expression of SOD1 and catalase genes. The promoter region-CAT fusion plasmids of SOD1 and catalase genes SODCAT and the pBluCLCAT, respectively, were introduced into HepG2 cells. The TS and PT treatments had no effect on either SOD1 or catalase gene expression, but 100 μg/mL of PD induced the transcription of both SOD1 and catalase genes about 2–3 fold, respectively (Fig.2).

We then examined the major subfraction of PD to find which ginsenoside is responsible for the induction of the SOD1 and catalase genes. PD consisted of ginsenosides Rb_1, -Rb_2, -Rc, -Rd and some other very minor fractions. Figure 3 shows that ginsenoside Rc and ginsenoside Rd had no effect on either induction or inhibition, whereas Rb_2 was 2–3 fold more potent an activator than Rb_1 on

both SOD1 and catalase expression. Rb_2, when added at increasing concentrations up to 100 μM for SOD1 and 200 μM for catalase genes, resulted in a gradual increase of the CAT values (Fig. 4A). To characterize the induction profile for the time course incubation, the medium of transfected cells was removed and treated with 50 μM Rb_2 in PBS for 30 min. After the incubation, the cells were washed with PBS and then fresh growth medium was added. The induction profile of SOD1 reached a peak at 2 h after Rb_2 addition and then declined gradually (Fig. 4B). Catalase reached a peak after a relatively short period incubation time with Rb_2 and maintained the induction level up to 4 h (Fig. 4B). These results confirmed panaxadiol ginsenoside Rb_2 as a specific inducer of both enzymes.

Both Rb_1 and Rb_2 have the basic structure of 20(S)-protopanaxadiol. Rb_1 has four molecules of glucose in the sugar moiety, whereas Rb_2 has three molecules of glucose and one molecule of arabinose (Fig. 1). Even though there are only minor differences in the sugar moiety, it has been suggested that the action mechanism of the two agents is somewhat different (Yokozawa *et al.*, 1993). A previous report showed that the ginsenoside Rg_1 modulates tyrosine aminotransferase gene transcription through its influence on functional or cooperative interaction between glucocorticoid receptor and cAMP-mediated induction (Kang *et al.*, 1994). Considering difficulties of purification and limited quantity, these results suggest that PD itself as well as Rb_2 would serve as inducers of antioxidant enzymes and thus may play a role in scavenging the cellular radical oxygen.

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