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Enzymatic transformation of the major ginsenoside Rb2 to minor compound Y and compound K by a ginsenoside-hydrolyzing β-glycosidase from *Microbacterium esteraromaticum*

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Abstract The ginsenoside-hydrolyzing β-glycosidase (Bgp3) derived from *Microbacterium esteraromaticum* transformed the major ginsenoside Rb2 to more pharmacologically active minor ginsenosides including compounds Y and K. The *bgp3* gene consists of 2,271 bp encoding 756 amino acids which have homology to the glycosyl hydrolase family 3 protein domain. Bgp3 is capable of hydrolyzing beta-glucose links and arabinose links. HPLC analysis of the time course of ginsenoside Rb2 hydrolysis by Bgp3 (0.1 mg enzyme ml^{-1} in 20 mM sodium phosphate buffer at 40 °C and pH 7.0) showed that the glycosidase first hydrolyzed the inner glucose moiety attached to the C-3 position and then the arabinopyranose moiety attached to the C-20 position. Thus, Bgp3 hydrolyzed the ginsenoside Rb2 via the following pathway: Rb2 → compound Y → compound K.

Keywords β-Glycosidase · Ginsenoside Rb2 · Compound Y · Compound K

Introduction

Ginseng, the root of *Panax ginseng* C. A. Meyer, has been used for more than 2,000 years as a traditional medicine in East Asia to strengthen immunity, provide nutrition, and reduce fatigue. Ginsenosides are the principal components of ginseng that are responsible for its biological and pharmaceutical properties. Many previous studies have focused on the anticancer [2], antitumor [8], anti-inflammatory [14], antidiabetic [9], antihypertensive [16], anti-fatigue [6], and antioxidative activities [7] of ginsenosides. Over 180 ginsenosides have been purified, characterized, and classified [4]. They are classified into three groups: oleanane, protopanaxadiol (PPD), and protopanaxatriol (PPT). The PPD ginsenosides harbor three sugar moieties (glucopyranose, α-L-arabinopyranose, α-L-arabinofuranose) at C-3 and C-20 of the aglycon PPD. The minor ginsenosides, including ginsenosides F2, Rg3, Rh2, compound Y, compound K, Rg2, F1, and Rh1, can be produced by hydrolysis of sugar moieties from the major ginsenosides Rb1, Rb2, Rc, Rd, Re, and Rg1, which comprise more than 80 % of the total ginsenosides [11].

Ginsenoside Rb2 is one of the major components of ginseng, comprising 2–17 % of total ginsenoside in ginseng root [5]. The deglycosylated compound K, which is absent in ginseng root, reportedly induces tumor cell apoptosis, inhibits tumor metastasis, and restrains tumor invasion [12, 18, 19]. Its production has been achieved from ginsenoside Rb2 by using enzymatic methods including use of the β-glucosidase from *Fusobacterium* K-60 [1] and β-glucosidase from *Aspergillus niger* [3]. However, these enzymes exhibited low selectivity and the enzymes involved were unknown.

In this study, we report the cloning and expression of the *bgp3* gene which encodes a ginsenoside-hydrolyzing β-glycosidase derived from *Microbacterium esteraromaticum*. This enzyme effectively converted the ginsenoside Rb2 to compounds Y and K by hydrolyzing the inner glucose moiety attached to the C-3 position and arabinopyranose moiety attached to the C-20 position. The

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elemental formula of compound Y was $C_{41}H_{70}O_{12}$ (Mw 755) and compound K was $C_{36}H_{62}O_8$ (Mw 623) in ESI–MS.

Materials and methods

Materials

The pure ginsenosides Rb2, compound O, F2, compound Y, Rh2, and compound K were obtained from ginseng genetic resource bank at Kyung-Hee University (Yongin, Korea).

Cloning of β-glycosidase (*bgp3*) gene from *M. esteraromaticum*

Microbacterium esteraromaticum (KACC 16318, Korean Agricultural Culture Collection, Suwon, Korea) was isolated from a ginseng field. Genomic DNA from *M. esteraromaticum* was extracted using the genomic DNA extraction kit (Gene ALL, Korea). The gene-encoded β-glycosidase was amplified from a genomic DNA and used as template by polymerase chain reaction with *Pfu* DNA polymerase. The gene termed *bgp3* (GenBank accession number JN 603821) was amplified using the following primers (with *Nde*1 and *Eco*RV restriction sites in boldface): *bgp3*F (5′-C**CA TAT G**GA GCC CCA GAT GAC CAA-3′) and *bgp3*R (5′-CC**G ATA TC**T CAG GCG AAG ACG-3′). The amplified fragment was digested with *Nde*1 and *Eco*RV and inserted into pMAL-c5X to generate the maltose binding protein (MBP)–*bgp3* gene fusion. The Bgp3 protein expression construct was verified by DNA sequencing.

Purification of β-glycosidase (Bgp3)

Escherichia coli BL21 (DE3), transformed with recombinant pMAL–*bgp3*, was grown in Luria–Bertani (LB)–ampicillin medium at 37 °C to an optical density at 600 nm (OD_{600}) of 0.4; protein expression was induced by adding 0.5 mM isopropyl-β-D-thiogalactopyranoside (IPTG). Bacteria were incubated for additional 9 h at 28 °C and harvested by centrifuging at 5,000×*g* for 30 min at 4 °C.

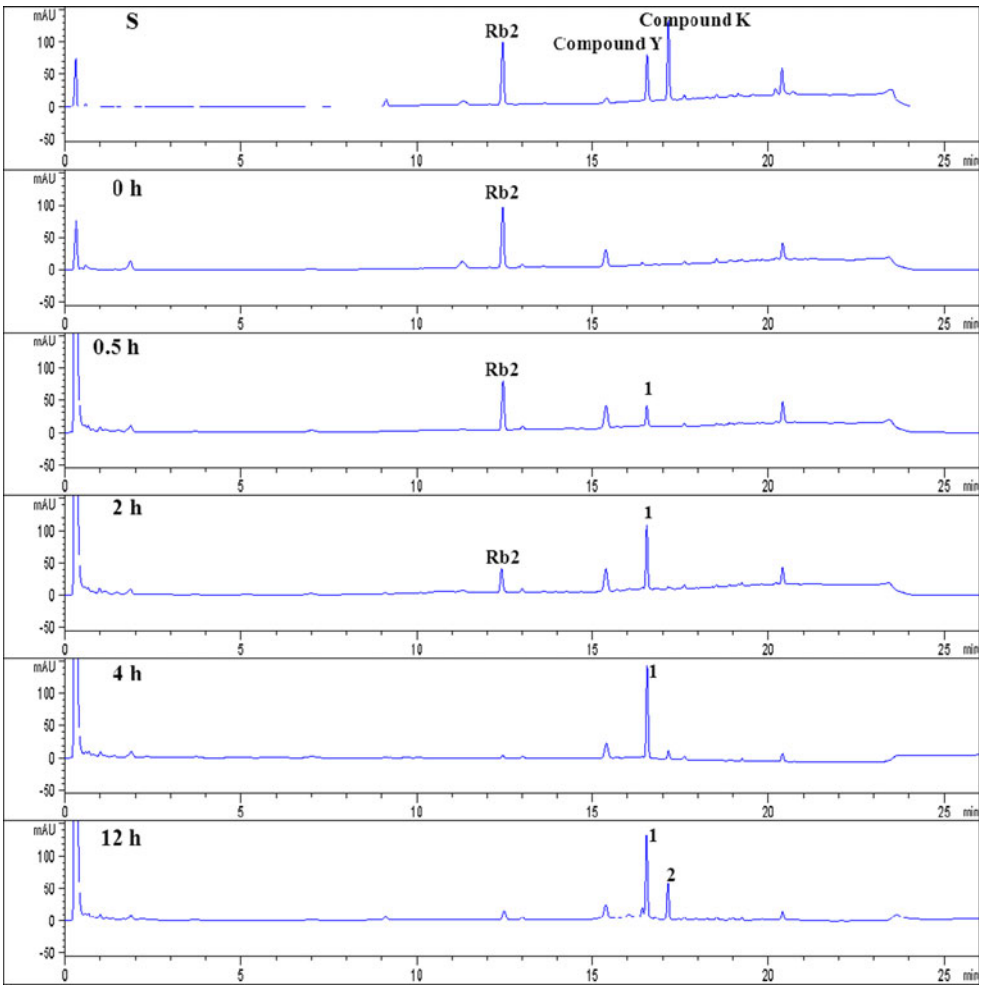

Fig. 1 HPLC analysis of the time course of the transformation of ginsenoside Rb2 by Bgp3. *S* ginsenoside standards, *1* metabolite 1, *2* metabolite 2

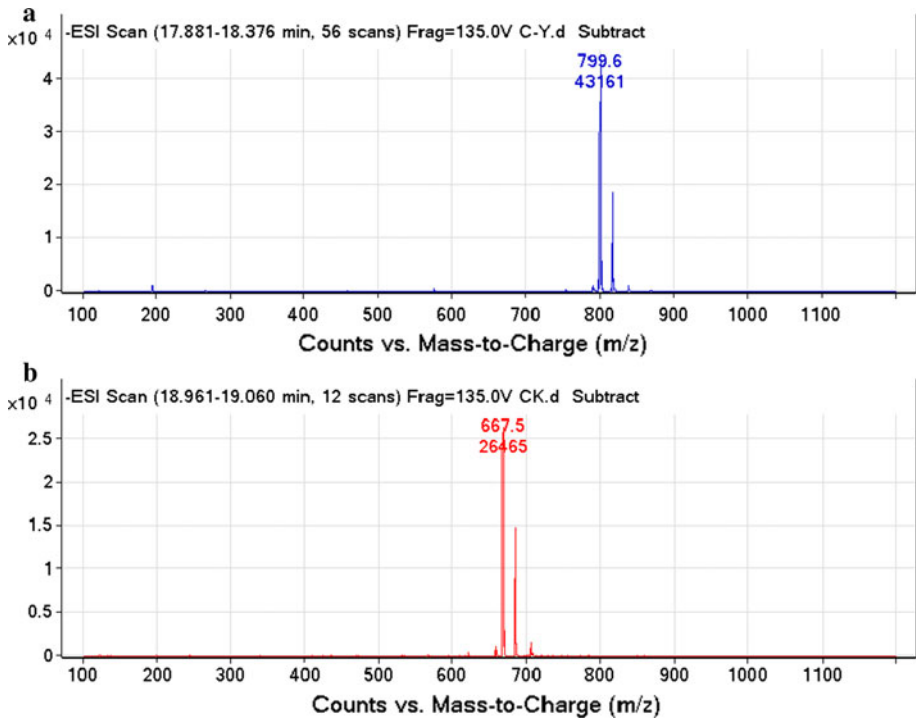

Fig. 2 Mass spectra of ginsenoside Rb2 after hydrolysis by Bgp3. **a** Mass spectrum of compound Y, *m/z* 800 = [Mw + formic acid], Mw 755. **b** Mass spectrum of compound K, *m/z* 668 = [Mw + formic acid], Mw 623

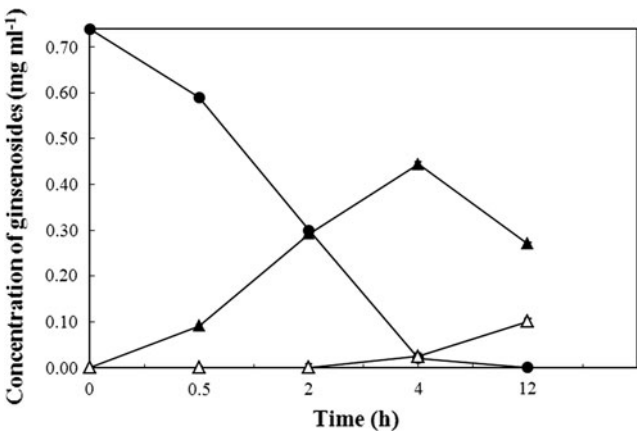

Fig. 3 Time course for compound Y and compound K production from ginsenoside Rb2 by Bgp3 at 40 °C and pH 7.0. Rb1 (*filled circle*) → compound Y (*filled triangle*) → compound K (*open triangle*)

The cells were washed twice with 20 mM sodium phosphate buffer (pH 7.0, 1 mM EDTA, and 1 mM NaCl) and then resuspended in 20 mM sodium phosphate buffer (pH 7.0). Cells were disrupted by 3-min periods of sonications at 1-s intervals with 5 repetitions on an ultrasonic processor at 70 % output with cooling on ice, and debris was removed by centrifugation (12,000×*g*, at 4 °C for 30 min). The crude extract of 5 ml cell lysate was applied to the pre-equilibrated amylose column (5 ml, NEB, USA), followed by washing with 12 column volumes of elution buffer (20 mM sodium phosphate buffer pH 7.0, 1 mM EDTA, and 1 mM NaCl). The bound proteins were eluted with elution buffer supplemented with 10 mM maltose. The cleaved proteins were applied to the second amylose column, and Bgp3 was eluted with flow through and purified. Protein concentration was determined using the SMART BCA protein assay kit (Intron, Korea).

Enzymatic hydrolysis of ginsenosides

The enzyme (Bgp3) at 0.1 mg ml^{-1} dissolved in 20 mM sodium phosphate buffer (pH 7.0) was incubated with an equal volume of ginsenosides Rb2, compound O, F2, and compound Y at 1.0 mg ml^{-1} in 20 mM sodium phosphate buffer (pH 7.0) at 40 °C. Samples were withdrawn at regular intervals. An equal volume of water-saturated *n*-butanol was added to each sample to stop the reaction; subsequently, the *n*-butanol fraction was evaporated to dryness, and the methanol extract was analyzed by TLC, HPLC, and LC/MS.

TLC analysis of ginsenosides

TLC was performed with silica gel plates ($60F_{254}$, Merck, Darmstadt, Germany), with the developing solvent $CHCl_3$/CH_3OH/H_2O (65:35:10). Spots on the TLC plates were detected by spraying plates with 10 % H_2SO_4 followed by heating at 110 °C for 10 min.

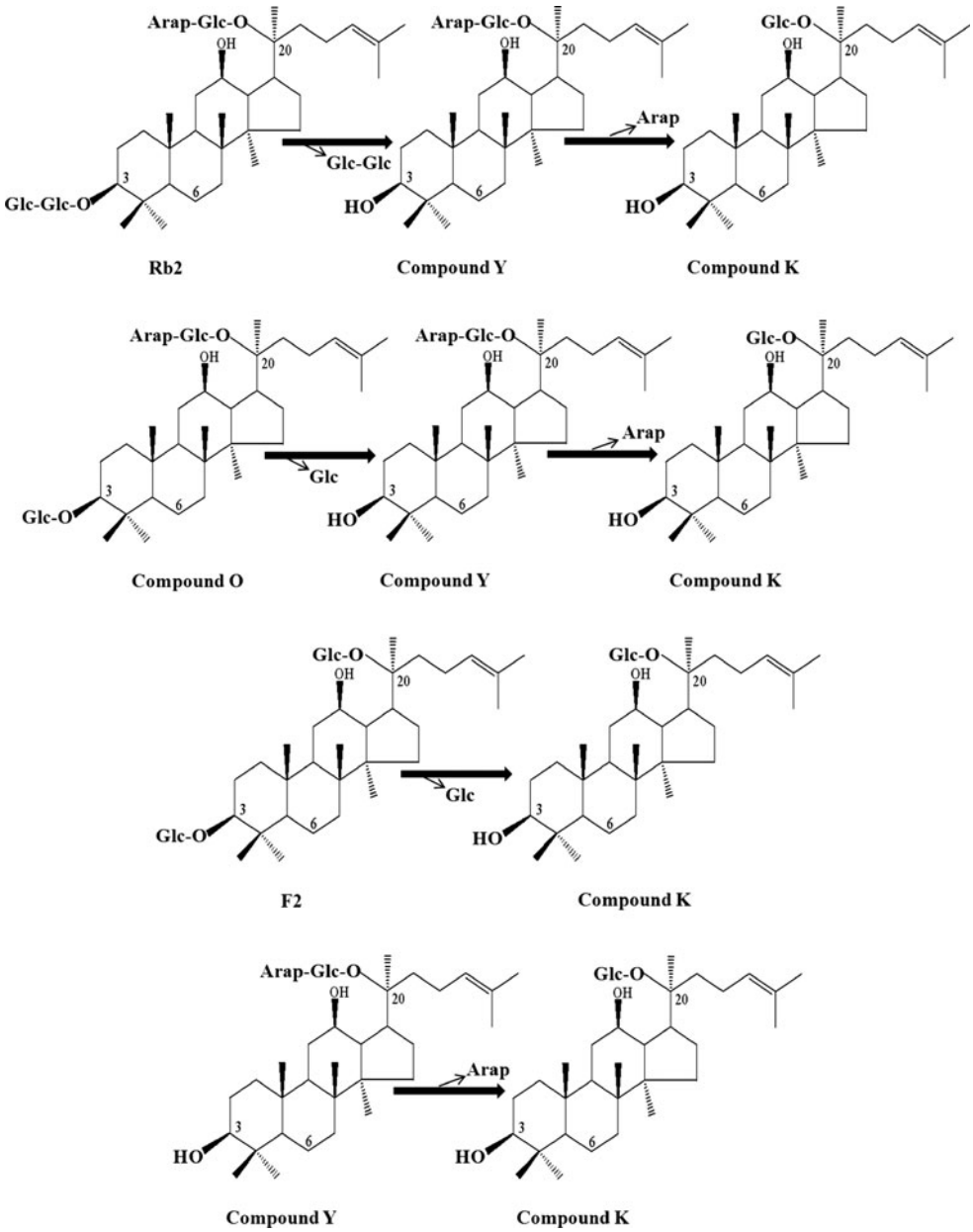

Fig. 4 Transformation pathway of ginsenoside Rb2, compound O, F2, and compound Y by Bgp3

Analysis of ginsenosides by HPLC and LC/MS

The reaction mixture was extracted with *n*-butanol saturated with H_2O, and evaporated in a vacuum. The residue was dissolved in methanol and analyzed by HPLC using a C_{18} (250 × 4.6 mm, particle size 5 μm) column with acetonitrile (solvent A) and distilled water (solvent B) as mobile phases at 85 % B for 5 min, 79 % B for 20 min, 42 % B for 55 min, 10 % B for 12 min, and 85 % B for 18 min all at 1.6 ml min^{-1}. Detection was at 203 nm. For LC/MS, ginsenosides were analyzed by Agilent QQQ/MS with positive polarity and an ion trap analyzer. Ion spray was operated under 5 l N_2/min, 3.5 kV, 25 psi, and 300 °C.

Results and discussion

Biotransformation pathway of ginsenoside Rb2 by Bgp3

A gene consisting of 2,271 bp encoding 756 amino acids, which have homology to the protein domain of glycosyl hydrolase families 3 [17] and with the same sequence as *bgp3* (GenBank accession number JN 603821), was cloned and expressed in *E. coli.* To maximize the yield of the fusion protein we tested different conditions for protein induction. Induction with 0.5 mM IPTG at 28 °C for 9 h produced the maximum level of soluble active fusion enzyme. The MBP–Bgp3 fusion protein was purified from

cell-free lysate by affinity chromatography on amylose resin. The time-course experiment was performed and the hydrolyzed product was periodically analyzed by HPLC (Fig. 1). Hydrolysis of the ginsenoside Rb2 produced two different metabolites: ginsenoside Rb2 was transformed to an intermediate, metabolite 1, and then to the final product, metabolite 2. The retention times of metabolites 1 and 2 on HPLC were similar to those of compounds Y and K.

Metabolites 1 and 2 from the transformation were subjected to LC/MS to determine their molecular weights (Mw). Metabolite 1 was obtained as a white powder and displayed a pseudomolecular ion peak [Mw + formic acid] at *m/z* 800 in ESI–MS, corresponding to the elemental formula $C_{41}H_{70}O_{12}$ (Mw 755). Thus, metabolite 1 was confirmed as being compound Y (Fig. 2a).

The molecular formula of metabolite 2 was determined to be $C_{36}H_{62}O_8$ based on the protonated molecular ion peak [Mw + formic acid] at *m/z* 668 (Mw 623) in ESI–MS. This result confirmed that metabolite 2 was compound K (Fig. 2b).

The conversion of ginsenoside Rb2 by Bgp3 was confirmed quantitatively by HPLC analysis. As shown in Fig. 3, 0.74 mg ml^{-1}ginsenoside Rb2 was transformed to 0.27 mg ml^{-1}compound Y and 0.1 mg ml^{-1} compound K after 12 h. Hence, Bgp3 hydrolyzed the ginsenoside Rb2 along the following pathway Rb2 → compound Y → com pound K, i.e., it hydrolyzed the inner glucose moiety attached to the C-3 position, followed by the arabinopyranose moiety attached to the C-20 position of ginsenoside Rb2 (Fig. 4).

Substrate specificity of Bgp3

Theoretically, four sugar moieties attached to the ginsenoside Rb2 could be available for hydrolysis by Bgp3, namely, the outer and inner glucose moieties attached at position C-3, and arabinopyranose or glucose moieties attached at position C-20. On the basis of the HPLC analysis of the hydrolysis products of ginsenoside Rb2, Bgp3 first hydrolyzed the inner glucose moiety attached to the C-3 position of ginsenoside Rb2 and then the arabinopyranose moiety attached to the C-20 position, effectively converting Rb2 to compounds Y and K.

To investigate whether Bgp3 showed the same specificity and selectivity for glycone residues attached at the C-3 and C-20 positions of other PPD ginsenosides, the ginsenosides F2, compound O, and compound Y were also used as substrates (Fig. 5). Compound O was converted to compounds Y and K by hydrolysis of the glucose moiety at position C-3 and the arabinopyranose moiety at position C-20, whereas ginsenoside F2 was converted to compound K by hydrolysis of the glucose moiety at position C-3. When compound Y was used as a substrate, Bgp3

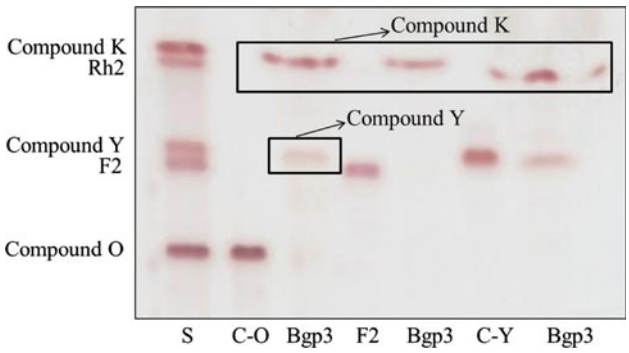

Fig. 5 Transformation of ginsenosides F2, compound O, and compound Y by Bgp3. Metabolites were analyzed by TLC. *C-O* compound O, *C-Y* compound Y

hydrolysis of the arabinopyranose moiety at the C-20 position of compound Y produced compound K (Fig. 4). The ginsenoside-hydrolyzing α-L-arabinopyranosidase from *Bifidobacterium breve* K-110 [13] exploits the hydrolytic pathway Rb2 → Rd; β-D-glycosidase from *Thermus caldophilus* [15] exploits the hydrolytic pathway Rb2 → Rd; cell-free extracts from *Bifidobacterium* sp. Int57 [3] exploit the hydrolytic pathway Rb2 → Rd → F2 → compound K; and β-glycosidase from *Sulfolobus acidocaldarius* [10] exploits the hydrolytic pathway Rb2 → compound Y → compound K.

Conclusions

This study demonstrated that compounds K and Y were produced from the ginsenoside Rb2 by Bgp3, a ginsenoside-hydrolyzing β-glycosidase from *M. esteraromaticum.* The recombinant β-glycosidase was able to convert ginsenoside Rb2 into rare ginsenosides through selective hydrolysis of glucose and arabinopyranose moieties, yielding compounds Y and K. Therefore the Bgp3 enzyme is considered potentially useful for the practical preparation of compounds Y and K.

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References

1. Bae EA, Park SY, Kim DH (2000) Constitutive beta-glucosidases hydrolyzing ginsenoside Rb1 and Rb2 from human intestinal bacteria. Biol Pharm Bull 23(12):1481–1485
2. Chae S, Kang KA, Chang WY, Kim MJ, Lee SJ, Lee YS, Kim HS, Kim H, Hyun JW (2009) Effect of compound K, a metabolite of ginseng saponin, combined with gamma-ray radiation in human lung cancer cells in vitro and in vivo. J Agric Food Chem 57:5777–5782

3. Chi H, Kim DH, Ji GE (2005) Transformation of ginsenosides Rb2 and Rc from *Panax ginseng* by food microorganisms. Biol Pharm Bull 28:2102–2105
4. Christensen LP (2008) Chapter 1 ginsenosides: chemistry, biosynthesis, analysis, and potential health effects. Adv Food Nutr Res 55:1–99
5. Hu C, Kitts DD (2001) Free radical scavenging capacity as related to antioxidant activity and ginsenoside composition of Asian and North American ginseng extracts. J Am Oil Chem Soc 78:249–255
6. Lee HU, Bae EA, Han MJ, Kim NJ, Kim DH (2005) Hepatoprotective effect of ginsenoside Rb1 and compound K on *tert*-butyl hydroperoxide-induced liver injury. Liver Int 25:1069–1073
7. Liu ZQ, Luo XY, Liu GZ, Chen YP, Wang ZC, Sun YX (2003) In vitro study of the relationship between the structure of ginsenoside and its antioxidative or pro oxidative activity in free radical induced hemolysis of human erythrocytes. J Agric Food Chem 51:2555–2558
8. Mochizuki M, Yoo YC, Matsuzawa K, Sato K, Saiki I, Tono-oka S, Samukawa K, Azuma I (1995) Inhibitory effect of tumor metastasis in mice by saponins, ginsenoside-Rb2, 20(*R*)- and 20(*S*)-ginsenoside-Rg3, of red ginseng. Biol Pharm Bull 18:1197–1202
9. Ni HX, Yu NJ, Yang XH (2010) The study of ginsenoside on PPARγ expression of mononuclear macrophage in type 2 diabetes. Mol Biol Rep 37:2975–2979
10. Noh KH, Oh DK (2009) Production of the rare ginsenosides compound K, compound Y, and compound Mc by a thermo stable beta-glycosidase from *Sulfolobus acidocaldarius*. Biol Pharm Bull 32(11):1830–1835
11. Noh KH, Son JW, Kim HJ, Oh DK (2009) Ginsenoside compound K production from ginseng root extract by a thermo stable β-glycosidase from *Sulfolobus solfataricus*. Biosci Biotechnol Biochem 73:316–321
12. Oh SH, Yin HQ, Lee BH (2004) Role of the Fas/Fas ligand death receptor pathway in ginseng saponin metabolite-induced apoptosis in HepG2 cells. Arch Pharm Res 27:402–406
13. Shin HY, Park SY, Sung JH, Kim DH (2003) Purification and characterization of α-L-arabinopyranosidase and α-L-arabinofuranosidase from *Bifidobacterium breve* K-110, a human intestinal anaerobic bacterium metabolizing ginsenoside Rb2 and Rc. Appl Environ Microbiol 69:7116–7123
14. Song X, Zang L, Hu S (2009) Amplified immune response by ginsenoside-based nanoparticles (ginsomes). Vaccine 27:2306–2311
15. Son JW, Kim HJ, Oh DK (2008) Ginsenoside Rd production from the major ginsenoside Rb1 by beta-glucosidase from *Thermus caldophilus*. Biotechnol Lett 30:713–716
16. Stavro PM, Woo M, Heim TF, Leiter LA, Vuksan V (2005) North American ginseng exerts a neutral effect on blood pressure in individuals with hypertension. Hypertension 46:406–411
17. Varghese JN, Hrmova M, Fincher GB (1999) Three-dimensional structure of a barley beta-D-glucan exohydrolase, a family 3 glycosyl hydrolase. Structure 15:179–190
18. Wakabayashi C, Hasegawa H, Murata J, Saiki I (1997) In vivo antimetastatic action of ginseng protopanaxadiol saponins is based on their intestinal bacterial metabolites after oral administration. Oncol Res 9:411–417
19. Wakabayashi C, Murakami K, Hasegawa H, Murata J, Saiki I (1998) An intestinal bacterial metabolite of ginseng protopanaxadiol saponins has the ability to induce apoptosis in tumor cells. Biochem Biophys Res Commun 246:725–730