Marked Production of Ginsenosides Rd, F_2, Rg_3, and Compound K by Enzymatic Method[1)]

Sung-Ryong Ko,*[,a] Yukio Suzuki,[b] Kei Suzuki,[b] Kang-Ju Choi,[a,2)] and Byung-Goo Cho[a]

[a] *K T & G Central Research Institute; 302 Shinseong-dong, Yuseong-ku, Daejeon 305–805, Korea: and* [b] *Okayama University; 6–45 Iwami-cho, Kurashiki 710–0814, Japan.*

Received May 2, 2007; accepted July 16, 2007; published online July 25, 2007

The hydrolysis of protopanaxadiol-type saponin mixture by various glycoside hydrolases was examined. Among these enzymes, crude preparations of lactase from *Aspergillus oryzae*, β-galactosidase from *A. oryzae*, and cellulase from *Trichoderma viride* were found to produce ginsenoside F_2 [3-*O*-(β-D-glucopyranosyl)-20-*O*-β-D-glucopyranosyl-20(*S*)-protopanaxadiol], compound K [20-*O*-β-D-glucopyranosyl-20(*S*)-protopanaxadiol], and ginsenoside Rd {3-*O*-[β-D-glucopyranosyl-(1→2)-β-D-glucopyranosyl]-20-*O*-β-D-glucopyranosyl-20(*S*)-protopanaxadiol}, respectively, from protopanaxadiol-type saponin mixture in large quantities. Moreover, the crude preparation of lactase from *Penicillium* sp. having a high producing activity of ginsenoside Rh_1 (6-*O*-β-D-glucopyranosyl-20(*S*)-protopanaxatriol) from protopanaxatriol-type saponin mixture gave ginsenoside Rd as a main product, ginsenoside Rg_3 {3-*O*-[β-D-glucopyranosyl-(1→2)-β-D-glucopyranosyl]-20(*S*)-protopanaxadiol}, and compound K from protopanaxadiol-type saponin mixture. The hydrolytic pathways of ginsenosides Rb_1, Rb_2, and Rc to ginsenosides Rd, Rg_3, and F_2, and compound K by crude preparations of four glycoside hydrolases were also studied. This is the first report on the enzymatic preparation of an intestinal bacterial metabolite, ginsenoside F_2, in quantity, and a considerable amount of a minor saponin, ginsenoside Rg_3, from a protopanaxadiol-type saponin mixture.

Key words ginsenoside F_2; ginsenoside Rg_3; compound K; *Penicillium* sp. lactase; *Aspergillus oryzae* lactase; *Aspergillus oryzae* β-galactosidase

During our investigations on glycosylation and hydrolysis of ginseng saponins by various glycoside hydrolases,[3—5)] a minor saponin, 20(*S*)-ginsenoside (G) Rh_1, was found to be produced in high yields (more than 90%) from major saponins such as G-Re and G-Rg_1 by a crude lactase preparation from *Penicillium* sp.[6)] Furthermore, we reported that not only G-Rh_1 but also G-Rg_2 (another minor saponin) and an intestinal bacterial metabolite such as G-F_1 were enzymatically prepared with high efficiency from protopanaxatriol-type (Ppt-type) saponin mixture that was readily obtained from ginseng extract.[7,8)] The present paper describes the enzymatic preparation of G-Rd, G-F_2, and an intestinal bacterial metabolite, compound K (C-K), in quantities, and of a considerable amount of G-Rg_3 from protopanaxadiol-type (Ppd-type) saponin mixture.

In a preliminary experiment, we screened a variety of commercially crude glycoside hydrolase preparations (such as ten cellulases, a hemicellulase, a β-glucosidase, three hesperidinases, two naringinases, three β-galactosidases, seven lactases, four pectinases, five α-amylases, two maltogenic α-amylases, two β-amylases, a glucoamylase, three pullulanases, a dextranase, and an isomaltase) for their activities in the production of minor saponins and intestinal bacterial metabolites from Ppd-type saponin mixture. HPLC profiles showed that crude preparations of cellulase from *Trichoderma viride* (Onozuka R-10) (Enz.C), lactase from *Aspergillus oryzae* (lactase F) (Enz.LF), and β-galactosidase from *A. oryzae* (Kohjin) (Enz.G) gave the most remarkable production of new peaks (tentatively named compounds I, III, IV), respectively, from Ppd-type saponin mixture (Fig. 1). These new peaks were not formed in the reaction mixture with boiled enzyme preparations. Enz.C completely hydrolyzed both G-Rb_1 and G-Rc, but weakly hydrolyzed G-Rb_2 in Ppd-type saponin mixture, and gave a marked accumulation of compound I having a retention time similar to that of G-Rd. Enz.LF converted almost all of G-Rb_1, G-Rb_2, G-Rc, and G-Rd in Ppd-type saponin mixture into a remarkable amount of compound III being similar in retention time to G-F_2, together with small amounts of two unknown compounds X_1 and X_2 (peaks X_1 and X_2) having shorter retention times than that of compound III. Nearly complete disappearance of G-Rb_1, G-Rb_2, G-Rc, and G-Rd in a Ppd-type saponin mixture, large production of compound IV showing a retention time consistent with C-K, minute quantity of compound III having a retention time corresponding to G-F_2, and small amounts of two unknown compounds X_3 and X_4 (peaks X_3 and X_4) showing shorter retention times than that of compound IV were observed in the incubated reaction mixture with Enz.G. Also, the crude preparation of lactase from *Penicillium* sp. (Enz.L) having a high activity in the production of G-Rh_1 from Ppt-type saponin mixture[8)] formed mainly compound I from Ppd-type saponin mixture, together with a considerable amount of compound II having a retention time very similar to that of G-Rg_3, a significant amount of compound IV showing a retention time consistent with compound K, and a small peak being similar in a retention time to G-F_2. These compounds I, II, III, and IV were isolated from the incubated reaction mixture on a semipreparative scale by extraction with *n*-butanol and column chromatography, and identified by spectroscopy (Fig. 2). Neither of compounds X_1, X_2, X_3, and X_4 could be isolated in sufficient amounts to be identified. In the ^{13}C-NMR data (Table 1) for these compounds I, III, and IV, all of the carbon signals due to the aglycone moiety appeared at very similar positions to those of G-Rb_1. Therefore, these compounds were assumed to be prosapogenins of G-Rb_1. By comparison of the ^{13}C-NMR spectrum for compound I with that for G-Rb_1, the number of sugar moieties decreased from four in G-Rb_1 to

* To whom correspondence should be addressed. e-mail: srko@ktng.com

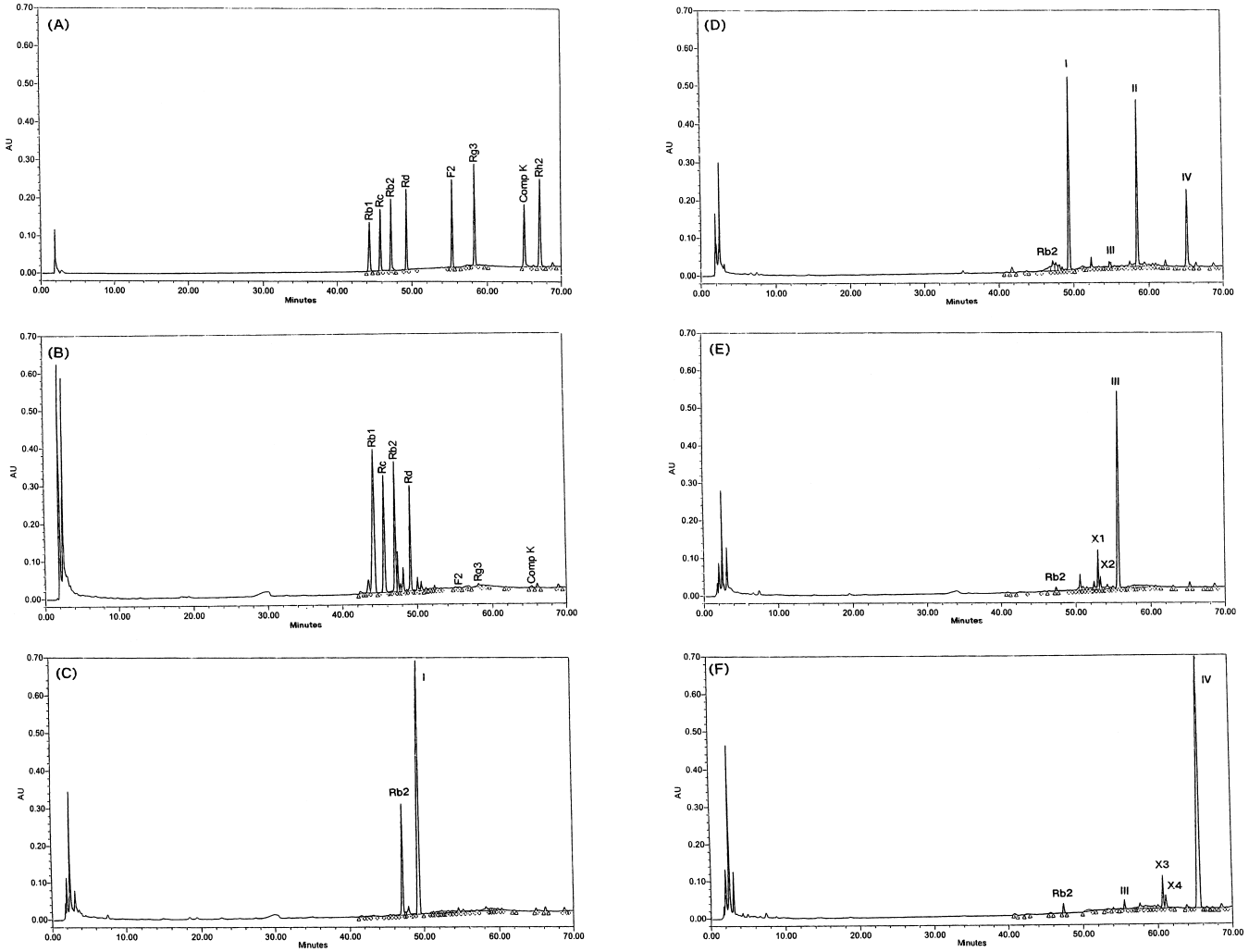


Fig. 1. HPLC Profiles of the Hydrolyzates of a Ppd-Type Saponin Mixture by Crude Preparations of Four Glycoside Hydrolases

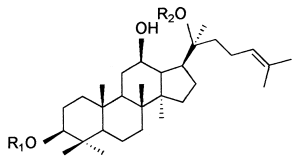
The reaction mixture containing 25 mg of a Ppd-type saponin mixture in 0.5 ml of methanol, 2.5 ml of 0.2 M sodium acetate buffer, 50 mg of enzyme preparation, and distilled water to make a final volume of 5.0 ml was incubated at 37 °C in the dark with gentle stirring. After a 72 h-incubation, each reaction mixture was extracted twice with 10 ml of *n*-butanol saturated with water, and then centrifuged. The *n*-butanol layer was washed twice with 4 ml of water, concentrated to dryness *in vacuo*, and the residue was dissolved in 2.5 ml of methanol. The methanol solution (20 μl) was applied on HPLC. Enzyme preparations and the pH of the buffer used in the reaction mixture were as follows. Enz.C: 4.5; Enz.L: 4.5; Enz.LF: 4.5; and Enz.G: 5.0. In the case of Enz.L, 125 mg of enzyme preparation was used. (A) A 20 μl of the mixture containing each of standard G-Rb_1, G-Rb_2, G-Rc, G-Rd, G-F_2, G-Rg_3, G-Rh_2, and C-K in the concentration of 500 ppm was applied on HPLC. (B) A 20 μl of 1% Ppd-type saponin mixture dissolved in methanol was applied on HPLC. The 1% Ppd-type saponin mixture contained G-Rb_1, G-Rc, G-Rb_2, G-Rd, G-F_2, G-Rg_3, and C-K in the concentration of 2325 ppm, 1229 ppm, 1152 ppm, 752 ppm, 12 ppm, 43 ppm, and 20 ppm, respectively. (C) A Ppd-type saponin mixture+Enz.C. (D) A Ppd-type saponin mixture+Enz.L. (E) A Ppd-type saponin mixture+Enz.LF. (F) A Ppd-type saponin mixture+Enz.G. Peaks I, II, III, and IV: compounds I, II, III, and IV.

three in compound I, whose anomeric carbon signals appeared at 98.1, 105.0, and 105.9 ppm. Moreover, based on three β-anomeric proton signals at 5.20, 4.92 and 5.38 ppm in the ^{1}H-NMR spectrum (in Experimental), compound I was identified as G-Rd, that is, 20(*S*)-protopanaxadiol having two glucose molecules in a sophorosyl structure linked at its C-3 hydroxyl group and one glucose molecule linked at its C-20 hydroxyl group. Compound III was identified as G-F_2, namely, 20(*S*)-protopanaxadiol having one glucose molecule linked at both its C-3 and C-20 hydroxyl groups, based on the ^{1}H- and ^{13}C-NMR data for compound III, in which the anomeric proton and carbon signals, respectively, appeared at 4.92 and 5.17, and 106.7 and 98.1 ppm. Compound IV was identified as compound K, *viz.*, 20(*S*)-protopanaxadiol having one glucose molecule linked at its C-20 hydroxyl group, based on the ^{1}H- and ^{13}C-NMR data for compound IV, in which the anomeric proton and carbon signals appeared at 5.20 and 98.2, respectively. Compound II was identified as G-Rg_3, that is, 20(*S*)-protopanaxadiol having a sophorose molecule linked at its C-3 hydroxyl group, based on the anomeric proton and carbon signals in ^{1}H- and ^{13}C-NMR spectra which appeared at 4.94 and 5.38, and 105.0 and 106.0 ppm, respectively. The values of carbon signals at C-10, C-11, C-17, C-21, and especially C-20 of compound II were different from those of the other three compounds having sugar molecule linked at the C-20 hydroxyl group of aglycone.

Figure 3 indicates the TLC profiles of the time course (0.5—24 h) in the hydrolysis of G-Rb_1, G-Rb_2, and G-Rc by the crude preparations of four enzymes. Enz.C readily hydrolyzed both G-Rb_1 and G-Rc into G-Rd, but very weakly G-Rb_2 into G-Rd, and also had no hydrolytic activity against G-Rd. It was observed that Enz.LF hydrolyzed mainly G-Rb_1 into G-F_2 *via* G-Rd, but G-Rb_2 and G-Rc into G-F_2 *via* compound V having a slightly higher *Rf* value than that of G-Rd in TLC and *via* compound VI having a slightly higher *Rf*

	R_1	R_2
G-Rb_1	-glc(2-1)glc	-glc(6-1)glc
G-Rb_2	-glc(2-1)glc	-glc(6-1)ara(pyr)
G-Rc	-glc(2-1)glc	-glc(6-1)ara(fur)
G-Rd (C-I)	-glc(2-1)glc	-glc
G-Rg_3 (C-II)	-glc(2-1)glc	-H
G-Rh_2	-glc	-H
G-F_2 (C-III)	-glc	-glc
C-K (C-IV)	-H	-glc
C-Mc_1	-glc	-glc(6-1)ara(fur)
C-Mc	-H	-glc(6-1)ara(fur)
C-O	-glc	-glc(6-1)ara(pyr)
C-Y	-H	-glc(6-1)ara(pyr)
Ppd	-H	-H

G: ginsenoside; C: compound; glc: β-D-glucopyranosyl; ara(pyr): α-L-arabinopyranosyl; ara(fur): α-L-arabinofuranosyl; Ppd: 20(*S*)-protopanaxadiol.



R_1=R_2=H: 20(*S*)-protopanaxadiol

Fig. 2. Chemical Structures of Ginsenosides and Their Related Compounds

value than that of compound V in TLC, respectively. It was assumed that Enz.LF also hydrolyzed a small part of G-Rb_1 into G-F_2 *via* compound IX having a slightly higher *Rf* value than that of G-Rd in TLC. Enz.G converted G-Rb_1 into C-K successively *via* G-Rd and G-F_2, and G-Rb_2 into C-K *via* compounds V and VII having a slightly higher *Rf* value that of G-F_2 in TLC. Enz.G was considered to convert G-Rc into C-K by two routes, one *via* compounds VI and VIII having a slightly higher *Rf* value than that of compound VII in TLC, and another *via* G-Rd and G-F_2. Enz.L hydrolyzed all of G-Rb_1, G-Rb_2 and G-Rc into G-Rd as a main product and G-Rg_3, and then a small part of G-Rd into C-K *via* G-F_2. These results showed that Enz.C and Enz.L had no hydrolytic activity against sophorose molecule linked at the C-3 hydroxyl group of aglycone in four ginsenosides (G-Rb_1, -Rb_2, -Rc, and -Rd), and three ginsenosides (G-Rb_1, -Rb_2, and -Rc), respectively. On the other hand, Enz.LF and Enz.G had a high hydrolytic activity toward sophorose molecule linked at the C-3 hydroxyl group of aglycone in G-Rb_2 and G-Rc, but not in G-Rb_1. From these results, the hydrolytic pathways of Ppd-type saponins by crude preparations of four glycoside hydrolases are suggested as follows:

Enz.C: G-Rb_1→G-Rd
G-Rb_2→G-Rd
G-Rc→G-Rd

Enz.L: G-Rb_1→G-Rd→G-Rg_3; G-Rb_1→G-Rd→G-F_2→C-K
G-Rb_2→G-Rd→G-Rg_3; G-Rb_2→G-Rd→G-F_2→C-K
G-Rc →G-Rd→G-Rg_3; G-Rc→G-Rd→G-F_2→C-K

Enz.LF: G-Rb_1→G-Rd→G-F_2; G-Rb_1→Compound IX

Table 1. ^{13}C-NMR Chemical Shifts of Compounds I, II, III, IV

	G-Rb_1	C-I	G-Rg_3	C-II	C-III	C-IV
Aglycone moieties						
C-1	39.1	39.0	39.5	39.3	38.9	39.3
C-2	26.6	26.6	27.3	27.0	26.5	28.2
C-3	89.3	88.8	89.2	88.8	88.7	78.2
C-4	39.6	39.5	40.3	39.9	39.5	39.5
C-5	56.3	56.2	56.8	56.4	56.1	56.3
C-6	18.6	18.3	18.7	18.3	18.2	18.7
C-7	35.1	35.0	36.1	35.8	34.9	35.1
C-8	39.9	39.9	37.2	36.8	39.8	40.0
C-9	50.1	50.0	50.7	50.3	49.9	50.2
C-10	36.8	36.7	39.9	39.6	36.7	37.3
C-11	30.8	30.7	32.2	32.0	30.6	30.7
C-12	70.1	70.1	71.2	70.9	70.1	70.1
C-13	49.3	49.3	48.9	48.5	49.5	49.4
C-14	51.3	51.3	51.9	51.6	51.2	51.4
C-15	30.8	30.7	31.6	31.2	30.4	30.9
C-16	26.6	26.5	26.9	26.8	26.4	26.6
C-17	51.6	51.5	54.9	54.7	51.5	51.5
C-18	16.2	16.2	16.7	16.5	16.1	16.3
C-19	15.9	15.8	16.4	16.3	15.8	16.0
C-20	83.5	83.4	73.2	72.9	83.2	83.2
C-21	22.6	22.3	26.9	26.6	22.3	22.3
C-22	36.1	36.0	35.5	35.1	35.8	36.1
C-23	23.1	23.1	23.2	22.9	23.1	23.1
C-24	125.8	125.8	126.5	126.2	125.7	125.9
C-25	131.0	130.8	130.8	130.7	130.8	130.8
C-26	25.8	25.6	25.8	25.7	25.6	25.7
C-27	17.9	17.6	17.7	17.6	17.6	17.7
C-28	28.0	27.9	28.3	28.0	27.9	28.6
C-29	16.5	16.5	16.0	15.7	16.6	16.3
C-30	17.3	17.2	17.3	16.9	17.1	17.3
Sugar moieties						
3-Glc						
1	105.0	105.0	105.0	105.0	106.7	
2	82.9	83.2	83.5	83.3	75.6	
3	77.2	77.8	78.1	78.2	78.8	
4	71.5	71.4	72.0	71.7	71.8	
5	78.0	78.0	77.8	77.8	78.3	
6	62.6	62.7	63.2	62.9	63.1	
Glc						
1	105.6	105.9	105.9	106.0		
2	76.7	77.0	76.9	77.1		
3	78.8	78.7	78.5	78.2		
4	71.5	71.5	72.1	71.8		
5	78.0	78.1	78.0	78.0		
6	62.6	62.8	63.2	62.9		
20-Glc						
1	97.9	98.1			98.1	98.2
2	74.9	75.0			75.0	75.0
3	78.0	78.6			79.0	79.3
4	71.5	71.5			71.7	71.6
5	76.7	78.1			78.5	78.0
6	71.5	62.6			62.7	62.8
Glc						
1	105.0					
2	74.9					
3	78.0					
4	71.5					
5	78.0					
6	62.6					

Glc: β-D-glucopyranosyl; G-Rg_3: 20(*S*)-G-Rg_3.

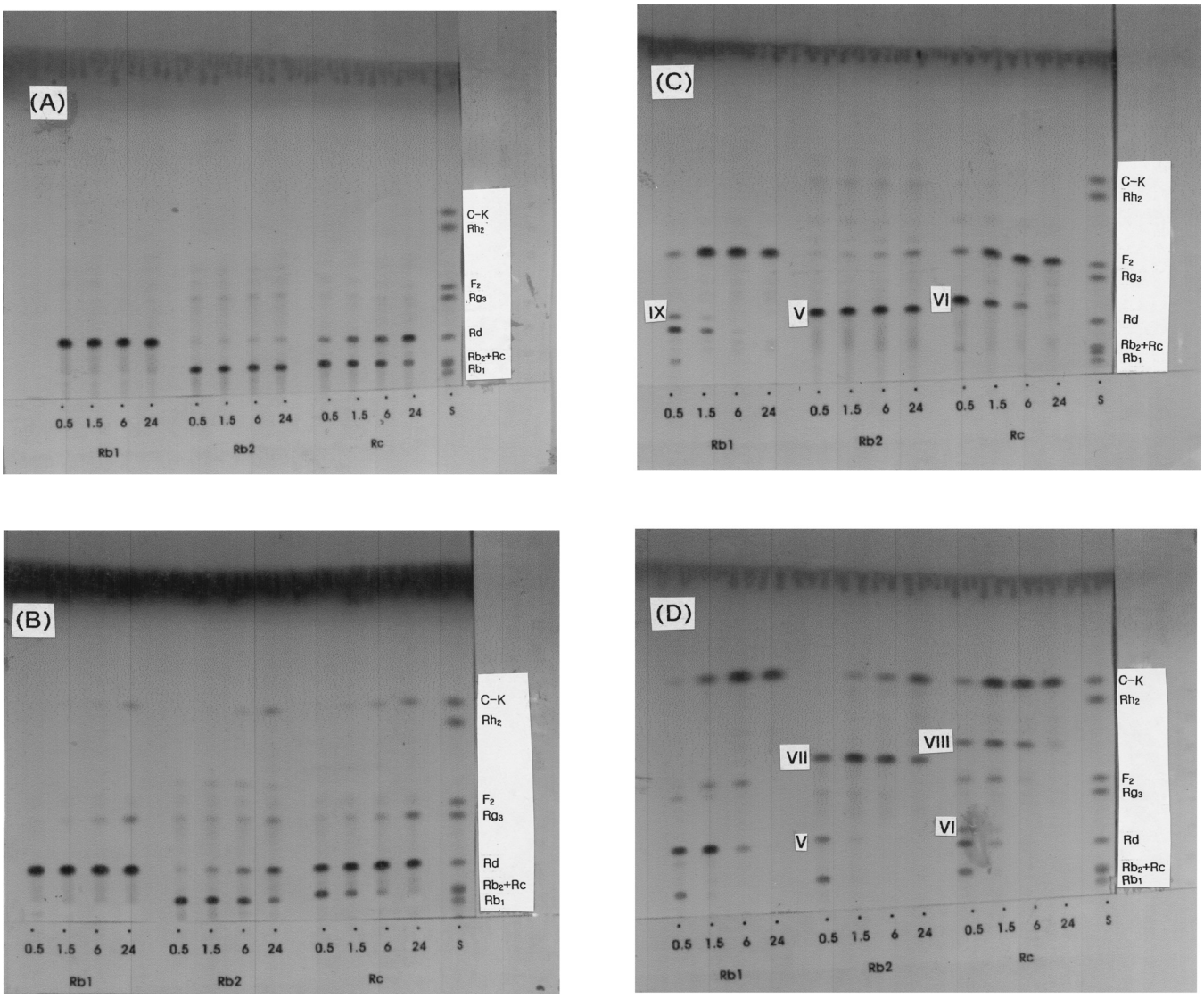


Fig. 3. Thin-Layer Chromatograms of Hydrolyzates of Three Ginsenosides Rb_1, Rb_2, and Rc by Crude Preparations of Four Enzymes

The reaction mixture containing 2 mg of G-Rb_1 (or G-Rb_2, G-Rc) in 0.25 ml of methanol, 4 mg of enzyme preparation, 1.25 ml of 0.2 M sodium acetate buffer, and distilled water to make a final volume of 2.5 ml was incubated for 0.5, 1.5, 6, and 24 h at 37 °C in the dark with gentle stirring. After each of incubation, the reaction mixture was extracted with 0.5 ml of *n*-butanol saturated with water and centrifuged. An aliquot (5 μl) of the *n*-butanol layer was applied on TLC. (A) G-Rb_1 (or G-Rb_2, G-Rc)+Enz.C; (B) G-Rb_1 (or G-Rb_2, G-Rc)+Enz.L; (C) G-Rb_1 (or G-Rb_2, G-Rc)+Enz.LF; and (D) G-Rb_1 (or G-Rb_2, G-Rc)+Enz.G. S: the mixture of G-Rb_1, G-Rb_2, G-Rc, G-Rd, G-Rg_3 G-F_2, G-Rh_2, and C-K in order of lower *Rf* values. Incubation time: 0.5, 1.5, 6, and 24 h. V, VI, VII, VIII, and IX: compounds V, VI, VII, VIII, and IX.

$\rightarrow$G-F_2
G-$Rb_2$$\rightarrow$Compound V$\rightarrow$G-$F_2$
G-Rc$\rightarrow$Compound VI$\rightarrow$G-F_2

Enz.G: G-$Rb_1$$\rightarrow$G-Rd$\rightarrow$G-$F_2$$\rightarrow$C-K
G-$Rb_2$$\rightarrow$Compound V$\rightarrow$Compound VII$\rightarrow$C-K
G-Rc$\rightarrow$G-Rd$\rightarrow$G-$F_2$$\rightarrow$C-K; G-Rc$\rightarrow$Compound VI $\rightarrow$Compound VIII$\rightarrow$C-K

Kohda and Tanaka reported on the hydrolysis of ginseng saponins with crude preparations of several glycoside hydrolases.[9] Emulsin and crude preparations of cellulase and amylase exhibited very low hydrolytic activities, while crude preparations of pectinase, naringinase, and hesperidinase, especially the last one, had high activities. The incubation of a mixture of G-Rb_1, G-Rb_2, and G-Rc with a crude hesperidinase from *A. niger* (Tanabe Seiyaku Co., Ltd., Osaka, Japan) gave C-K in *ca.* 70% yield. They also reported that the hydrolytic activity must be due to some other enzymes present in crude hesperidinase preparation, as purified hesperidinase did not hydrolyze the saponins. Koizumi *et al.* reported that G-Rb_2 afforded four prosapogenins such as G-F_2, C-K, C-O, and C-Y on stepwise hydrolysis with commercial naringinase (Sigma).[10] Recently, several investigators have reported that G-Rb_1, G-Rb_2, and G-Rc are metabolized by intestinal bacteria in rats and humans after oral administration and that the main metabolite of Ppd-type saponins is C-K.[11—13] The metabolic pathways of three ginsenosides by intestinal bacteria are as follows: G-$Rb_1$$\rightarrow$G-Rd$\rightarrow$G-$F_2$$\rightarrow$C-K; G-$Rb_2$$\rightarrow$C-O$\rightarrow$C-Y$\rightarrow$C-K; G-Rc$\rightarrow$C-$Mc_1$$\rightarrow$C-Mc$\rightarrow$C-K. This compound (C-K) has inhibited lung metastasis of melanoma cells and *in vitro* tumor cell invasion and migration at nontoxic or marginally toxic concentrations.[13]

In this work, 46 commercially available crude preparations of 15 glycoside hydrolases were screened for hydrolytic activity against Ppd-type saponin mixture. Five α-amylases, two β-amylases, emulsin, and all of three hesperidinases [containing one from *A. niger* (Tanabe Seiyaku Co.)] tested

had very low activities. Four cellulases, two naringinases, and three pectinases showed considerable activities. Furthermore, three crude enzyme preparations (Enz.C, LF, and G) were found to have very highly hydrolytic activities, to afford G-Rd, G-F_2, and C-K, respectively, in large quantities from Ppd-type saponin mixture. Enz.LF accumulated compound V and compound VI in quantities from G-Rb_2 and G-Rc, respectively, in the earlier stage of hydrolytic reaction. Enz.G also accumulated considerable amounts of compound VII and compound VIII from G-Rb_2 and G-Rc, respectively. The isolation and identification of these compounds V, VI, VII, VIII and IX will be presented in a subsequent paper. All of these three enzyme preparations had no hydrolytic activity toward the β-glucosidic linkage attached to the C-20 hydroxyl group of aglycone. On the other hand, Enz.L having a high activity in the production of G-Rh_1 from Ppt-type saponin mixture[8] hydrolyzed all of G-Rb_1, G-Rb_2, and G-Rc into G-Rd (main product) and a considerable amount of G-Rg_3, that is, Enz.L regioselectively hydrolyzed C-20-*O*-β-D-glucosidic linkage in G-Rd, to afford a minor saponin, G-Rg_3. These results reveal that the enzymatic method is very useful for preparing minor saponins and intestinal bacterial metabolites with selectivity and efficiency from ginseng saponin mixture.

Experimental

Chemical Reagents and Enzymes A Ppd-type saponin mixture containing G-Rb_1, G-Rb_2, G-Rc, and G-Rd, and each of these ginsenosides were obtained from standardized ginseng extract containing 13—14% of total saponin (K T & G Central Research Institute) by the reported methods.[14,15] G-F_2 was prepared from the leaves of the same plant (*Panax ginseng* C. A. MEYER, Araliaceae) by the reported procedures.[16] G-Rg_3 and G-Rh_2 were prepared by the acid hydrolysis of G-Rb_1 with 50% aqueous acetic acid at 70 °C, following the separation of the racemic mixture of 20(*R* and *S*)-G-Rg_3 into 20(*R*)-G-Rg_3 and 20(*S*)-G-Rg_3 by using acetylation and silica column chromatography, and further treatments of the acetylate of 20(*S*)-G-Rg_3 with 5% sodium hydroxide in *n*-butanol at room temperature for 1 h and at 40 °C for 2 h, respectively, according to the reported procedures.[17] The structures of these compounds are shown in Fig. 2. The purity of these compounds was assessed by TLC, HPLC, and ^{13}C-NMR spectroscopy. Crude preparations of four glycoside hydrolases, *i.e.*, a cellulase from *T. viride* (ONOZUKA R-10) (Enz.C) (Yakult Honsha Co., Ltd., Tokyo, Japan), a β-galactosidase from *A. oryzae* (Enz.G) (Kohjin Co., Ltd., Tokyo, Japan), two lactases from *A. oryzae* (lactase F) (Enz.LF) (Amano Pharmaceutical Co., Ltd., Nagoya, Japan) and *Penicillium* sp. (Enz.L) (KI Chemical Ind. Co., Ltd., Iwata, Shizuoka, Japan) were kindly supplied.

Analyses TLC: A silica gel G-60 F_{254} TLC plate (E. Merck); developing solvent, chloroform–methanol–water (65 : 35 : 10, v/v/v, lower phase) (solvent I); detection, 20% sulfuric acid in ethanol and heating (105—110 °C for 10 min). HPLC: pump, Waters 510; auto sampler, Waters 717 plus; data system, Waters Millenium 32; column, Discovery C_{18} (4.6×250 mm, 5 μm, Supelco Co.), column temperature, 30 °C; mobile phase, gradient conditions with water (solvent A) and acetonitrile (solvent B) were as follows: the ratios of solvent A/solvent B at running times of 0, 10, 40, 55, 70, 72, 82, 84, 90 min were 80/20, 80/20, 68/32, 50/50, 35/65, 10/90, 10/90, 80/20, and 80/20, respectively; flow rate, 1.6 ml/min; detector, ginsenosides eluted were detected at 203 nm of UV using Waters 2996 Photodiode Array detector. Melting point: a Fisher-Johns melting point apparatus. Optical rotation values: a JASCO DIT-370 polarimeter. FAB-mass: a JEOL mass spectrometer model JMS-HX-110/110A with a matrix of methanol and 3-nitrobenzyl alcohol (1 : 20, v/v). ^{1}H-NMR (400 MHz) and ^{13}C-NMR (100 MHz): a Burker spectrometer model AMX 400 in C_5D_5N with tetramethylsilane as an internal standard.

Isolation of Enzymatic Hydrolysis Products of Ppd-Type Saponin Mixture On a semipreparative scale, a reaction mixture containing 0.4 g of Ppd-type saponin mixture in 5 ml of methanol, 0.8 g of Enz. C [or LF, G, or 2 g of Enz.L] preparation, 10 ml of 0.2 M sodium acetate buffer (pH 4.5), and water to make a final volume of 50 ml was incubated for 72 h at 37 °C. After incubation, two volumes of methanol were added to the reaction mixture, and the mixture was adjusted to pH 6.5, heated for 10 min in a boiling water bath, and centrifuged. The supernatant was concentrated below 37 °C *in vacuo*. The concentrate was extracted several times with *n*-butanol, and centrifuged. The *n*-butanol layer was concentrated to dryness *in vacuo*, and dissolved in a small amount of methanol. The methanol solution obtained from the reaction mixture with Enz.C was chromatographed on a silica gel 60 (230—400 mesh) column (60 g, column ϕ=2.5 cm) developed with acetonitrile–water–2-propanol (80 : 5 : 15, v/v/v), and rechromatographed on silica gel 60 column (60 g, column ϕ=2.5 cm) with chloroform–methanol–ethyl acetate–water (2 : 2 : 4 : 1, v/v/v/v, lower phase) to afford compound I (176 mg) which was identified as G-Rd from its TLC, HPLC, ^{1}H- and ^{13}C-NMR spectra (Table 1). Each methanol solution from the reaction mixture with Enz.L, LF, or G was respectively applied on the following 1st and 2nd column chromatography: silica gel 60 column with chloroform–methanol (7 : 1,v/v) and LiChroprep RP-8 column with methanol–water (8 : 2, v/v); silica gel 60 column with chloroform–methanol–water (50 : 8 : 1, v/v/v), and polyamide column using gradient elution with methanol–water (1 : 15, v/v—1 : 5, v/v); and silica gel 60 column with chloroform–methanol–water (7 : 3 : 0.5, v/v/v) and LiChroprep RP-8 column using 80—85% methanol as eluent. Compounds II, III, and IV were respectively obtained at 70, 124, and 109 mg from Ppd-type saponin mixture by the hydrolytic action of Enz.L, LF, and G. The purity of these compounds was assessed by *Rf* values on TLC and retention time in HPLC analyses. These compounds were identified by comparison of their FAB-MS, and ^{1}H- and ^{13}C-NMR data with those of authentic samples.

Compound I: Colorless fine crystals from 2-propanol; mp 206—208 °C; $[\alpha]_D^{22}$ +19.4° (*c*=1.03, methanol); *Rf* value, 0.35 on a Kieselgel 60 F_{254} TLC plate developed with solvent I; FAB-MS, *m/z* 948 $(M+1)^+$; ^{1}H-NMR (δ), three anomeric proton signals due to β-glucosidic linkages at 4.92 (1H, d, *J*=7.42 Hz, H-1 of inner glucose at C-3 of aglycone), 5.20 (1H, d, *J*=7.62 Hz, H-1 of glucose at C-20 of aglycone), and 5.38 (1H, d, *J*=7.56 Hz, H-1 of terminal glucose at C-3 of aglycone); ^{13}C-NMR (Table 1).

Compound II: Colorless fine crystals from 90% isopropanol; mp 248—250 °C; $[\alpha]_D^{24}$ +16.5° (*c*=1.00, methanol); *Rf* value, 0.49 on a Kieselgel 60 F_{254} TLC plate developed with solvent I; FAB-MS, *m/z* 786 $(M+1)^+$; ^{1}H-NMR (δ), two anomeric proton signals due to β-glucosidic linkages at 4.94 (1H, d, *J*=7.50 Hz, H-1 of inner glucose at C-3 of aglycone), and 5.38 (1H, d, *J*=7.62 Hz, H-1 of terminal glucose at C-3 of aglycone); ^{13}C-NMR (Table 1).

Compound III: Colorless fine crystals from *n*-butanol; mp 186—188 °C; $[\alpha]_D^{20}$ +29.6° (*c*=1.01, methanol); *Rf* value, 0.53 on a Kieselgel 60 F_{254} TLC plate developed with solvent I; FAB-MS, *m/z* 807 $[M+Na]^+$; ^{1}H-NMR (δ), two anomeric proton signals due to β-glucosidic linkages at 4.92 (1H, d, *J*=7.02 Hz, H-1 of glucose at C-3 of aglycone), and 5.17 (1H, d, *J*=7.27 Hz, H-1 of glucose at C-20 of aglycone); ^{13}C-NMR (Table 1).

Compound IV: White amorphous powders from ethanol; mp 176—178 °C; $[\alpha]_D^{22}$ +9.1° (*c*=0.89, chloroform); *Rf* value, 0.68 on a Kieselgel 60 F_{254} TLC plate developed with solvent I; FAB-MS (negative), *m/z* 621 $[C_{36}H_{62}O_8\text{-H}]^-$, 459 $[C_{36}H_{62}O_8\text{-Glc-H}]^-$; ^{1}H-NMR ($\delta$), one anomeric proton signal due to β-glucosidic linkage at 5.20 (1H, d, *J*=7.76 Hz, H-1 of glucose at C-20 of aglycone); ^{13}C-NMR, (Table 1). These compounds I, II, III, IV were identified respectively as 3-*O*-[β-D-glucopyranosyl-(1→2)-β-D-glucopyranosyl]-20-*O*-β-D-glucopyranosyl-20(*S*)-protopanaxadiol (G-Rd), 3-*O*-[β-D-glucopyranosyl-(1→2)-β-D-glucopyranosyl]-20(*S*)-protopanaxadiol (G-Rg_3), 3-*O*-(β-D-glucopyranosyl)-20-*O*-β-D-glucopyranosyl-20(*S*)-protopanaxadiol (G-F_2), and 20-*O*-β-D-glucopyranosyl-20(*S*)-protopanaxadiol (C-K).

Acknowledgments This work was done at the KT & G Central Research Institute and supported in part by a Korea Science and Technology Foundation brain pool project research fellowship to one of the authors, Yukio Suzuki.

References and Notes

1) The complete title of this paper is "Enzymatic Preparation of Minor Saponins and Intestinal Bacterial Saponin Metabolites of Ginseng. Part III." The abstract of this paper was presented at the poster session of the 9th Int. Ginseng Symp., Geumsan in Korea, Sep. 25—28, 2006, p. 159.
2) Present address: *Pankorea Co., Ltd.; 458–7 Gung-dong, Yuseong-ku, Daejeon 305–335, Korea.*
3) Suzuki Y., Ko S. R., Choi K. J., Uchida K., Lee Y. G., Kim Y. H., Proc. 7th Int. Ginseng Symp., Seoul, 1998, pp. 373—374.
4) Kim Y. H., Lee Y. G., Choi K. J., Uchida K., Suzuki Y., *Biosci.*

Biotechnol. Biochem., **65**, 875—883 (2001).
5) Ko S. R., Suzuki Y., Kim Y. H., Choi K. J., *Biosci. Biotechnol. Biochem.*, **65**, 1223—1226 (2001).
6) Ko S. R., Suzuki Y., Choi K. J., Kim Y. H., *Biosci. Biotechnol. Biochem.*, **64**, 2739—2743 (2000).
7) Ko S. R., Choi K. J., Uchida K., Suzuki Y., *Planta Med.*, **69**, 285—286 (2003).
8) Ko S. R., Choi K. J., Suzuki K., Suzuki Y., *Chem. Pharm. Bull.*, **51**, 404—408 (2003).
9) Kohda H., Tanaka O., *Yakugaku Zasshi*, **95**, 246—249 (1975).
10) Koizumi H., Sanada S., Ida Y., Shoji J., *Chem. Pharm. Bull.*, **30**, 2393—2398 (1982).
11) Karikura M., Miyase T., Tanizawa H., Taniyama T., Takino Y., *Chem. Pharm. Bull.*, **39**, 2357—2361 (1991).
12) Kanaoka M., Akao T., Kobashi K., *J. Traditional Med.*, **11**, 241—245 (1994).
13) Wakabayashi C., Hasegawa H., Murata J., Saiki I., *Oncology Res.*, **9**, 411—417 (1997).
14) Sanada S., Kondo N., Shoji J., Tanaka O., Shibata S., *Chem. Pharm. Bull.*, **22**, 421—428 (1974).
15) Sanada S., Kondo N., Shoji J., Tanaka O., Shibata S., *Chem. Pharm. Bull.*, **22**, 2407—2412 (1974).
16) Yahara S., Tanaka O., Komori T., *Chem. Pharm. Bull.*, **24**, 2204—2208 (1976).
17) Baek N. I., Kim S. I., Lee Y. H., Kim D. S., Park J. D., Lee C. B., Proc. 6th Int. Ginseng Symp., Seoul, 1993, pp. 132—137.