Conversion of ginsenosides by *Lactobacillus plantarum* studied by liquid chromatography coupled to quadrupole trap mass spectrometry

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Abstract

Ginsenosides are the active components responsible for the pharmacological properties of ginseng, a commonly used medicinal plant and food ingredient. This study aimed to determine the changes of ginsenosides during fermentation of ginseng extract or reference ginsenosides with *Lactobacillus plantarum*. Chemically acidified ginseng extracts served as controls. High performance liquid chromatography coupled with quadrupole-trap (Q-TRAP) mass spectrometry method was employed for analysis and quantification of ginsenosides, and for identification of metabolites. A total of 14 metabolites were identified; the quantification of metabolites was achieved by tandem mass spectrometry in MRM mode. Metabolism of *L. plantarum* removed glucosyl moieties from ginsenosides Rb1, Rd, and Re at the C-20 position to produce a racemic mixture of products. Remarkably, removal of glysosyl residues occurred not only by hydrolysis but also by dehydration to produce racemic mixtures of Δ20(21) or Δ20(22) products. Biotransformation occurred more rapidly with the di-substituted ginsenoside Rb1 when compared to the mono-substituted ginsenoside Rd. This study thus extends the knowledge of biotransformation of ginsenosides to produce bioactive derivatives.

Keywords: ginsenoside, *Lactobacillus plantarum*, fermentation, biotransformation, Q-TRAP MS

Introduction

Ginseng is classified in the genus *Panax* of the *Araliaceae* family and has a long history of use in Chinese traditional medicine (Shibata, Fujita, Itokawa, Tanaka, & Ishii, 1963; Xiang, Shang, Gao, & Zhang, 2008). Pharmacological activities of ginseng are mainly attributed to ginsenosides (Niu et al., 2012), which are categorized according to the aglycone as protopanaxadiol (PPD), protopanaxatriol (PPT) and oleanolic acid. About 50 of ginsenoside variants have been identified (Angelova et al., 2008; Zhu, Li, Hau, Jiang, Yu, &Fong, 2011.) (Figure1). Ginsenosides Rg3 and Rg2 consist of *S* and *R* optical isomers depending on the orientation of the hydroxyl group on the chiral carbon C-20 (Cheng, Na, Bang, Kim, &Yang, 2008). Optical isomers exhibit different biological activities; the 20(*S*) isomer is more water-soluble (Kim et al., 2006). A racemic mixture of 20(*S*)- and 20(*R*)-Rg3 was readily produced from ginsenosides Rb1 or Rd through either acid treatment or heating (Han et al., 1982).

Recent studies on the bioavailability of ginsenosides suggest that bacterial metabolites formed during digestion contribute to pharmacological effects. Ginsenoside conversion by human intestinal microbiota proceeds mainly through deglycosylation (Hasegawa, Sung, Matsumiya, &Uchiyama, 1996; Wang, Hua, Liu, Liu, &Yu, 2014). Metabolites formed by the stepwise deglycosylation are more readily absorbed (Ruan, Leong, Yan, &Wang, 2010).

Ginsenosides are also transformed by microbial or enzymatic conversion during preparation of functional food ingredients (Chi &Ji, 2005). Studies on the microbial conversion of ginsenosides focused on food-grade bacteria, particularly lactobacilli

(Chi &Ji, 2005; Kim, Min, Quan, Lee, Yang, & Yang, 2012). Lactobacilli have a safe history of use in food fermentations and are suitable for fermentation of ginseng to achieve conversion of ginsenosides during food preparation (Chi &Ji, 2005; Kim et al., 2012). For example, lactobacilli converted ginsenoside Re by partial hydrolysis of glycosyl moieties to yield Rg2 and Rh1 as major products; conversion of Rb1 yielded Rd, F2, Rg3, Rh2, gypenoside XVII, or compound K (Chi &Ji, 2005; Kim, Choi, Kim, Suh, & Park, 2010; Kim et al., 2012; Figure 1).

Owing to the diversity of ginsenosides and because of the physiological and taxonomic diversity of food fermenting lactobacilli, the characterization of microbial pathways of conversion of ginsenosides during food fermentations remains insufficient. In particular, observations obtained in fermentation of plant material were not sufficiently verified by observation of extracts or reference compounds under sterile conditions. Moreover, kinetic analyses are unavailable. This study aimed to characterize the biotransformation of ginsenosides by *Lactobacillus plantarum* FUA3171. *L. plantarum* FUA 3171 was selected because of its ability to convert phenolic compounds in sorghum by glycosyl hydrolases, esterases, and other enzymatic activities (Sekwati Monang & Gänzle, 2011; Svensson, Sekwati Monang, Lutz, Schieber, & Gänzle, 2010). Liquid chromatography coupled with tandem mass spectrometry (LC-MS/MS) was employed as a powerful tool to achieve identification and quantification with high sensitivity and selectivity (Cui, M., Song, F. R., Zhou, Y., Liu, Z. Q., & Liu, S. Y., 2000; Angelova et al., 2008; Wu, Qin, Guo, Sun, & Liu, 2012; Li, Yang, Zhang, & Li, 2014; Wu, Sun, Zhang, Guo, & Liu, 2015). A highly

sensitive and selective liquid chromatography/quadrupole trap mass spectrometry (LC-Q-TRAP-MS) method (Svensson et al., 2010; Hu, Stromeck, Loponen, Lutz, Schieber, & Gänzle, 2011) was adapted to characterize and quantify metabolites of ginsenosides.

MATERIALS AND METHODS

Chemicals and Reagents. The following ginsenosides were used as reference: 20(*S*)-Re, 20(*S*)-Rg2, 20(*S*)-Rb1, 20(*S*)-Rd, 20(*S*)-Rg3, 20(*S*)-Rh2 (Sigma, Oakville, Canada). Acetonitrile, water and formic acid (95%) were HPLC-grade (Fisher Scientific, Ottawa, Canada).

Bacterial Growth and Media. *L. plantarum* FUA 3171 was grown on modified MRS (mMRS) agar for 24 h at 30 °C in modified atmosphere (1% O_2, 5% CO_2, 10% H_2, and balance N_2) and subcultured in modified MRS broth for 24 h at 30 °C. Modified MRS had the following composition per liter: 5.0 g of fructose, 5.0 g of glucose, 10.0 g of maltose, 10.0 g of tryptone, 5.0 g of beef extract, 5.0 g of yeast extract, 2.6 g of H_2KPO_4, 4.0 g of HK_2PO_4, 3.0 g of NH_4Cl, 0.5 g of L-cysteine HCl, 0.2 g of $MgSO_4$, 0.05 g of $MnSO_4$, 1.0 g of Tween 80, and 1.0 mL of vitamin mix (50 mg L^{-1} each of B12, B1, B2, B6, folic acid, and pantothenic acid). Solid media additionally contained 15 g L^{-1} agar.

Bacterial conversion of ginseng extract. Ground North American ginseng was obtained from a local supermarket. Extracts were prepared by immersing 10 g ground ginseng in 50 mL 70% methanol for 1 h, followed by processing with high intensity

ultrasound for 20 min. The supernatant were filtered and the residue was extracted again. Supernatants were pooled and methanol was evaporated under vacuum at 40 °C. The aqueous concentrate was extracted twice with 40 mL *n*-butanol, *n*-butanol was evaporated under vacuum at 50 °C, and solids were redissolved in 12 mL 80% methanol (Kim et al., 2010). Two mL of ginseng extract were evaporated to dryness, re-dissolved in 950 µL mMRS, inoculated with 50 µL of an overnight culture of *L. plantarum* FUA3171, and incubated for 48 h at 30 °C. The concentration of ginsenosides in MRS was thus approximately equivalent to the concentration in ginseng. To account for changes of ginsenosides in the absence of microbial activity, ginseng extracts were incubated for 48 h at 30 °C in sterile mMRS acidified to a pH of 4.0 with lactic acid and acetic acids in a ratio of 4:1 (v/v). Fermented ginseng exacts were characterized before and after fermentation by measuring the pH and determining the viable cell counts. Fermentations were carried out in two independent experiments. After fermentation, cells were removed by centrifugation and the supernatant was collected for LC-MS analysis.

Metabolism of reference ginsenosides by *Lactobacillus plantarum* FUA 3171 in mMRS. Reference ginsenosides 20(*S*)-Rb1, 20(*S*)-Rd, 20(*S*)-Rg3, 20(*S*)-Re and 20(*S*)-Rg2 were dissolved to a concentration of 5 g L^{-1} in methanol; 50 µL of these stock solutions were added to 400 µL mMRS media and inoculated with 50 µL of an overnight culture of *L. plantarum*. After 48 h of fermentation at 30 °C, cells were removed by centrifugation and supernatants were collected for LC-MS analyses to identification of ginsenosides.

Identification of ginsenosides by tandem MS. Ginsenosides were identified after adaptation of liquid chromatography-tandem mass spectrometry methods that was previously established for identification of phytochemicals from sorghum (Svensson et al., 2010; Bai, Findlay, Sanchez-Maldonado, Schieber, Vederas, & Gänzle, 2015). A Shimadzu UFLC system comprising a degasser, binary pump, autosampler, thermostated column compartment, and a SPD-M20A Prominence diode array (PDA) detector was connected to a 4000 Q TRAP LC-MS/MS System (MDS SCIEX, Applied Biosystems, Streetville, ON, Canada). Ginsenosides were separated on a Luna C18 RP-HPLC column (5 μm, 250×4.6mm, Phenomenex, Torrance, CA). The UV detection wavelength was set at 203nm, and diode array scanning was from 190 to 400 nm. Mobile phase A consisted of 0.1% (v/v) formic acid in water, and mobile phase B consisted of 0.1% (v/v) formic acid in acetonitrile. Samples were eluted with the following gradient: 0-10 min, 20% B; 10-15 min, 20-30% B; 15-20 min, 30% B; 20-23 min, 30-40% B; 23-27 min, 40% B; 27-45 min, 40-65% B; 45-50min, 65% B; 50-60 min, 65-85% B; 60-65 min, 85% B; 65-70 min, 85-20% B; 70-75 min, 20% B. The injection volume was 10 μL, and the flow rate was 0.8 mL min^{-1} at 35 °C.

Mass spectra were recorded in the negative-ion mode and the positive-ion mode; the values of operating parameters were as follows: spray voltage, -4500 V in negative mode and 5500 V in positive mode; source temperature, 600 °C; nebulizing gas (GS1), 65 psi; heating gas (GS2), 35 psi; and curtain gas, 12 psi. An information-dependent acquisition (IDA) method, EMS→3EPI, was used to identify ginsenosides. Both Q1 and Q3 were operated at unit mass resolution. The spectra were obtained over a range

from *m/z* 100 to 1800 in 0.8 s. LIT fill time was set at 20 ms. The IDA threshold was set at 500 cps, above which enhanced product ion (EPI) spectra were collected from the three most intense peaks. The EPI scan rate was 4000 amu s^{-1}. Collision-induced dissociation (CID) spectra were acquired using nitrogen as the collision gas under Rolling Collision Energy (CE, CE = 0.050 * (*m/z*) + 5.000 for negative-ion mode, and CE = 0.058 * (*m/z*) + 9.000 for positive-ion mode). The data analysis was performed by Analyst 1.5 (Applied Biosystems).

Quantification of 20(*S*)-Rg3 by MRM mode. Quantification of 20(*S*)-Rg3 was performed by multiple reaction monitoring (MRM) in positive-ion mode using external standards method (Hu et al., 2011) under LC conditions described above. The selection of the product and precursor ions was based on the IDA method outlined above; for quantification of 20(*S*)-Rg3 in MRM mode, the sodium adduct ion (*m/z* 807.7) was used as precursor ion, and the dominant fragment ion (*m/z* 365.5) was used as product ion. The parameters of MRM mode for quantification were as follows: declustering potential (DP), 197 V; entrance potential (EP), 10 V; collision energy (CE), 75 V; collision cell exit potential (CXP), 11 V. The values for optimum ion source parameters were as described above. External calibration standards were prepared in 50% (v/v) methanol, and the calibration curve of 20(*S*)-Rg3 was established with five concentrations ranging from 0.5 to 25 mg L^{-1}. The calibration curve was linear over the entire concentration range and the correlation coefficient r^2 was $\geqslant$ 0.99**.** The concentrations of the calibration curve encompassed all concentrations of 20(*S*)-Rg3 in fermented samples. Analysis of unfermented

microbiological media and media without addition of ginsenosides verified the absence of matrix effects interfering with the quantification.

To elucidate the kinetics of formation of 20(*S*)-Rg3 during microbial conversion of 20(*S*)-Rb1 and 20(*S*)-Rd, eight parallel samples consisting of 50 µL stock solution with 0.5 g L^{-1} ginsenosides 20(*S*)-Rb1 or 20(*S*)-Rd, 450 µL mMRS medium and 50µL overnight culture of *L. plantarum* FUA3171 were fermented at 30 °C. The fermentations were stopped after 18, 24, 48, 60, 72, 96, 120 or 144 h, respectively, by freezing the samples at -80 °C fridge. The samples were filtered through 0.22 µm Nylon membrane before quantification of 20(*S*)-Rg3 by LC-MRM. Duplicate parallel experiments were carried out for quantification of 20(*S*)-Rg3.

Results

Structural characterization of ginsenoside Re. The nomenclature for fragmentation of ginsenosides is based on Domon & Costello, 1988, Perreault & Costello, 1994, and Liu, Cui, Liu, & Song, 2004. Ions retaining the charge at the reducing terminus are termed Y and Z (glycoside cleavages) and X (cross-ring cleavages), and ions retaining the charge at the non-reducing terminus are termed B and C (glycoside cleavages) and A (cross-ring cleavages). As an example, the fragmentation patterns of ginsenoside 20(*S*)-Re in the negative-ion mode and positive-ion mode by Q-TRAP MS/MS are shown in Figure 2. Figure 2a shows the MS/MS spectrum of the *m/z* 945.5 ion ($[M-H]^-$) of Re in negative-ion mode. Under low collision energy conditions, the fragments can be assigned to Y and Z ions due to successive or simultaneous losses of

sugar moieties. There are six main fragment ions at *m/z* 799.4, 783.8, 765.8, 637.6, 619.6 and 475.6. The mass differences between the precursor ion (*m/z* 945.5) and the fragment ions *m/z* 799.4 ($Y_{1\beta}$) and *m/z*783.8 ($Y_{0\alpha}$) are 146 and 162 Da, indicating the losses of a deoxyhexose sugar and a hexose sugar, respectively (Cui et al., 2000). The fragment ion at *m/z* 637.6 ($Y_{0\beta}$/$Y_{1\beta'}$) matches the simultaneous loss of a deoxyhexose and a hexose. The smallest fragment ion at *m/z* 475.6 ($Y_{0\beta'}$) corresponds to the molecular weight of deprotonated panaxatriol, the type aglycone of Re (Figure 2c). Figure 2b shows the MS/MS spectrum of the *m/z* 969.6 ion ($[M+Na]^+$) of ginsenoside 20(*S*)-Re in the positive-ion mode. The fragment ions at *m/z* 789.1 ($Z_{0\alpha}$) and *m/z* 643.7 ($Z_{0\beta}$) correspond to the neutral losses of a hexose sugar moiety and a disaccharide comprising a dexoyhexose and a hexose sugar. The ion at *m/z* 349.4 ($C_{2\beta}$) matches the disaccharide adduct of the sodium ion. The characteristic fragment ions at *m/z* 441, 423 and 405 correspond to the PPT-type aglycone in positive ion mode (Qi, Wang, Zhang, Wang ,Li,& Yuan,2012).

Identification of ginsenosides in nonfermented and fermented ginseng extract. Separation and structural characterization of ginsenosides were achieved by LC-PDA-MS (Online Supplemental files S1). Both positive-ion and negative-ion modes were employed to obtain comprehensive data for identification and metabolite assignment. An information dependent acquisition (IDA) method was developed to identify ginsenosides in the extractions of nonfermented, fermented and chemically acidified ginseng extracts. External standards were analyzed for identification by comparison of retention time and mass spectrum under the same conditions. Literature

data of mass spectra and MS/MS fragmentation patterns were used for compounds for which external standards were not available. Due to the presence of formic acid in the mobile phase, the typical solvent adducts $[M+HCOO]^-$ and deprotonated molecular ions $[M-H]^-$ were usually detected in the negative-ion mode. Retention times, mass spectra, MS/MS fragmentation patterns in both negative-ion mode and positive-ion mode of the identified ginsenosides are listed in Table 1.

For the unfermented ginseng extract, a total of 15 major ginsenosides were identified based on comparison with external standards or tentatively identified based on comparison with literature data. For example, peak 16, a major ginsenoside in ginseng extracts, had the same elemental composition and produced similar MS/MS spectra as peak 13 (ginsenoside Rd), and was thus identified as gypenoside XVII, an isomer of ginsenoside Rd that eluted later on C18 columns (Wan, Liu, Wang, Qi, Wang, Li, & Yuan, 2013; Gafner, Bergeron, McCollom, Cooper, McPhail, Gerwick, & Angerhofer, 2004).

To exclude confounding effects of endogenous enzyme activities or background microbiota, fermentation experiments were carried out with ginseng extract in mMRS rather than ground ginseng. Prior to strain selection, growth and metabolic activity of *L. plantarum* in ginseng was verified. In ground ginseng mixed with water at a ratio of 1:1, *L. plantarum* FUA3171 grow to 9.66 ± 0.17 log(cfu mL^{-1}) after 24 h and acidified the fermentation substrate to pH 4.03 ± 0.01 by production of 212 ± 13 mmol kg^{-1} lactic acid. During fermentation of mMRS broth supplemented with ginseng extract with *L. plantarum*, the pH decreased 1 to 3.7 and the cell counts increased to 2.5 ×

10^8 cfu·mL^{-1} after 48 h of fermentation. A total of 14 metabolites were identified, which were not detected, or detected only in trace amounts in ginseng exact. Generally, metabolites shifted to longer retention times, reflecting the loss of hydrophilic glycosyl residues (Figure S1). Metabolites 9, 21 and 29 were identified as ginsenoside 20(*S*)-Rg2, ginsenoside 20(*S*)-Rg3 and ginsenoside 20(*S*)-Rh2 with reference compounds. Metabolites 11, 12, 18, 20, 22, 23, 24, 25, 26, 27 and 28 were assigned as ginsenoside 20(*R*)-Rg2, ginsenoside 20(*S*)-Rh1, ginsenoside Rg6, ginsenoside F4, ginsenoside 20(*R*)-Rg3, ginsenoside 20(*S*)-Rs3, ginsenoside 20(*R*)-Rs3, ginsenoside Rk1, Rs3-Δ20(21)H_2O, ginsenoside Rg5 and Rs3-Δ20(22)H_2O, respectively, based on molecular weight and fragmentation patterns. Chemical acidification of mMRS broth supplemented with ginseng extract did not alter the chromatographic profile of ginsenosides, demonstrating that the types of ginsenosides were changed by microbial activity.

Metabolism of ginsenosides reference compounds. To determine the metabolic activity of ginsenosides, culture media containing the pure ginsenosides 20(*S*)-Rb1, 20(*S*)-Rd, 20(*S*)-Rg3, 20(*S*)-Re and 20(*S*)-Rg2 as substrates were fermented for 48h with *L. plantarum* FUA 3171. An overview on the metabolites formed by the strain is provided in Figures 3 and 4. The conversion of 20(*S*)-Rb1 generated the metabolites 20(*S*)-Rg3 and 20(*R*)-Rg3, which are produced by hydrolysis of the C-20 sugar chain (Kwon, Han, Park, Kim, Park, & Park, 2001). The geometric isomers Rk1 and Rg5 were also identified; there are formed via dehydration and elimination of the C-20 sugar chain. The 20/21 double bond Rk1 elutes earlier than the 20/22-isomer Rg5

(Figure 3a and Figure 4a) (Qi,Wang,& Yuan, 2010). Ginsenosides F2 and Rd were not detected, indicating that substitutions at the C-3 were not hydrolysed, and that the two glucosyl residues at the C-20 position were eliminated simultaneously rather than by successive hydrolysis (Chen et al., 2008).

The conversion of ginsenosides 20(*S*)-Rd by *L. plantarum* is shown in Figure 3b and Figure 4b. The metabolites of 20(*S*)-Rd were 20(*S*)-Rg3, 20(*R*)-Rg3, Rk1 and Rg5, indicating an identical spectrum of metabolites independent on the number of glucosyl substitutions at the carbon 20. To determine whether dehydration and elimination of the glucosyl residue at the C-20 are simultaneous or sequential processes, 20(*S*)-Rg3 was offered as substrate for *L. plantarum*; however, this ginsenoside was not further modified (Figure 3c).

The conversion of 20(*S*)-Re is shown in Figures 3d and 4c, respectively. In analogy to 20(*S*)-Rd, 20(*S*)-Re is modified by hydrolysis of the glycosyl residue at C-20 to form ginsenosides 20(*S*)-Rg2 and 20(*R*)-Rg2, or *via* a dehydration and elimination process to form ginsenosides Rg6 and F4. To confirm that dehydration and elimination as simultaneous reactions, fermentations with the ginsenoside 20(*S*)-Rg2 as substrate were carried out. Metabolites of 20(*S*)-Rg2 were not detected (Figure 3e), confirming that *L. plantarum* catalyses simultaneous dehydration and elimination.

Kinetics of formation of ginsenoside 20(*S*)-Rg3 during fermentation of 20(*S*)-Rb1 and 20(*S*)-Rd. Ginsenoside 20(S)-Rg3 was formed by hydrolysis of a glucose residue from 20(S)-Rd, or by hydrolysis of a disaccharide from 20(S)-Rb1 (Compare Fig. 4a and 4b). To determine whether the length of the saccharide chain influences the

kinetics of formation of 20(*S*)-Rg3, ginsenosides 20(*S*)-Rb1 (containing two glucoses at the C-20 position) and 20(*S*)-Rd (containing one glucose at the C-20 position) were fermented with *L. plantarum* and samples were taken during 6 days of fermentation (Figure 5). Both substrates supported the formation of ginsenoside 20(*S*)-Rg3, however, the reaction proceeded more rapidly with 20(*S*)-Rb1 as substrate.

Discussion

The biological activity of ginseng is partially derived from bacterial metabolites of ginsenosides, which are formed by intestinal microbiota during digestion (Hasegawa et al., 1996; Ruan et al., 2010; Wang et al., 2014). The metabolism of ginsenosides by intestinal microbiota proceeds mainly by hydrolysis of glycosyl moieties at the C-3 and C-20 (Qian, Jiang, &Cai 2006; Yang, Deng, Xu, & Zeng, 2007); dehydrated metabolites including ginsenosides Rk1 and Rg5 and lactate esters of ginsenosides were also observed (Wang et al., 2014). The current study employed a highly sensitive and selective LC-Q-TRAP-MS method for analysis of ginsenosides and metabolites of ginsenoside standards to provide direct evidence for the microbial dehydration of gingenosides, and to analyse the kinetics of conversion by *L. plantarum*.

Fermentation of ginseng or ginseng extract allows controlled bioconversion of ginsenosides to more bioavailable and bioactive metabolites, and may provide opportunities for development of functional fermented foods and pharmacologically active preparations (nutraceuticals). Several studies provide proof of concept that fermented ginseng has improved biological activity. Fermented ginseng rich in ginsenosides Rg3, Rh1, and protopanaxatriol reduced the volume and weight of

hepatic tumors in rats (Lin, Mou, Su, &Chiang, 2010). Extract from ginseng fermented with *L. plantarum* was suggested to have superior immune-modulatory effects in human volunteers when compared to extract from non-fermented ginseng (Kim et al., 2011).

The conversion of ginsenosides during fermentation with lactic acid bacteria is attributable mainly to hydrolysis of glucosyl-residues at the C-3 and C-20 positions (Chi & Ji, 2005). This pathway or parts of this pathway has been described in several *Lactobacillus* species including *L. delbrueckii*, *L. pentosus, L. paralimentarius, L. ginsenosidimutans,* and *L. yonginensis*, and was attributed to β-glucosidase activity (Chi & Ji, 2005; Kim et al., 2012; Quan, Kim, Li, Choi, & Yang, 2013; Jung, Liu, Kim, Lee, Kim, & Im, 2013; Yi et al., 2013). Ginsenoside Rb1 was more readily transformed when compared to ginsenoside Re (Chi & Ji, 2005). A strain of *L. brevis* was recently reported to catalyse the exceptional ketonization of ginsenosides F1 and compound K (Jin et al., 2014).

The dehydrated ginsenosides Rk1 and Rg5 were moderately increased after fermentation of red ginseng with *L. plantarum* (Kim et al., 2010); however, because these metabolites were also produced during the heat sterilization of the fermentation substrate (Qi, Wang, & Yuan, 2010; Kim et al., 2010), evidence for their formation by microbial conversion is inconclusive. The current study used a ginseng extract and reference compounds as substrates for bacterial metabolism and thus excluded effects of heat treatment or endogenous (plant) enzymatic activities. This approach provided direct evidence for the formation of the dehydrated ginsenosides Rg5 and Rk1 by *L.*

323 *plantarum*. The production of dehydrated ginsenosides cannot be attributed to
324 glucosyl hydrolases; this conversion is thus attributable to yet uncharacterized
325 enzymatic activities of *L. plantarum*.

326 The pattern of metabolites formed during bioconversion of ginseng extract or pure
327 ginsenosides demonstrates that *L. plantarum* FUA3171 removes glucosyl-residues
328 mainly or exclusively at the C-20 position (Figure 4). Ginsenosides 20(*S*)-Rg3,
329 20(*R*)-Rg3, Rk1 and Rg5 may be produced by elimination of glucose chains at C-20
330 position of Rb1 and Rd (Figure 1 and Figure 4). 20(*S*)-Rg2, 20(*R*)-Rg2, Rg6 and F4
331 may be produced by the analogous conversion of ginsenoside Re (Figure 1).
332 Ginsenosides 20(*S*)-Rs3, 20(*R*)-Rs3, Rs3-Δ20(21)H_2O and Rs3-Δ20(22)H_2O may be
333 produced from quinquenoside R1 or pseudoginsenoside Rc1. Minor ginsenosides Rh2
334 may be produced from gypenoside XVII and F2. Because of the low concentration of
335 some ginsenosides, the *S* and *R* isomers of the minor ginsenosides Rh2 and Rh1 were
336 not differentiated.

337 This study is the first to provide a kinetic analysis of the conversion of ginsenosides
338 standards, which revealed two remarkable features. First, the ginsenoside Rb1 with
339 two glucosyl-residues at position C-20 was converted faster than ginsenoside Rd,
340 which carries only one glucosyl residue at C-20, and accumulation of ginsenoside Rd
341 was not observed during conversion of ginsenoside Rb1. In contrast to the sequential
342 hydrolysis of glycosyl-residues at position C-20 that was described for other
343 lactobacilli, this suggests the presence of a β-glucosidase which preferentially releases
344 the disaccharide. Second, the dehydrated ginsenosides Rk1, Rg5, Rg6, and F4 were

formed only from precursors that were glucosylated at the C-20 position (Figure 4). This conversion confirms that *L. plantarum* forms dehydrated ginsenosides, and may indicate the exceptional presence of a carbohydrate lyase in *L. plantarum*. To our knowledge, carbohydrate-lyases have not been described in *Lactobacillus* spp., and genes corresponding to carbohydrate lyases are not annotated in the genomes that are currently available at Genebank. Strains of *L. plantarum*, however, have an exceptional ability for conversion of plant secondary metabolites (Rodríguez et al., 2009). Moreover, when compared to other lactobacilli, *L. plantarum* is characterized by a large genome size and genome flexibility, including the ability to acquire "lifestyle cassettes" to occupy specific ecological niches (Siezen & van HylckamaVlieg, 2011). It is noteworthy that *L. plantarum* FUA3171 was isolated from sorghum fermentations, a substrate which is rich in phenolic compounds and glycosides of phenolic compounds (Sekwati-Monang & Gänzle, 2011).

In conclusion, this study assessed biotransformation of ginsenosides extracts by *Lactobacillus plantarum*. A total of 14 metabolites were characterized by LC-Q-TRAP-MS. Interestingly, deglycosylation and dehydration occurs predominantly at the C-20 position, and the elimination efficiency increases with the length of C-20 glucosyl-moieties. This study provided evidence for the exceptional existence of a glycosyl lyase in *L. plantarum* and will be useful for development of fermented ginseng products as functional foods or nutraceuticals.

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494

Figure legends

Figure 1. Chemical structures of ginsenosides and their metabolites.

Figure2. ESI-Q-TRAP-MS/MS spectra of ginsenoside 20(*S*)-Re. Panel (a): negative-ion mode. Panel (b): positive-ion mode. Panel (c): Fragmentation pathways of the $[M-H]^-$ ion of ginsenoside 20(*S*)-Re.

Figure 3. Extracted ion chromatograms (EICs) of metabolites formed by *L. plantarum* FUA3171 with standard ginsenosides as substrates. The following reference compounds were used as substrate: Panel (a) 20(*S*)-Rb1, Panel (b) 20(*S*)-Rd, Panel (c) 20(*S*)-Rg3, Panel (d) 20(*S*)-Re, Panel (e) 20(*S*)-Rg2.

Figure 4.Metabolic pathways of the conversion of ginsenosides 20(*S*)-Rb1 (Panel a) 20(*S*)-Rd (Panel b), and 20(*S*)-Re (Panel c).by *L. plantarum* FUA3171.

Figure 5. Kinetics of the formation of ginsenoside 20(*S*)-Rg3 during fermentation with *L. plantarum* FUA3171 and ginsenosides 20(*S*)-Rb1 or 20(*S*)-Rd as substrate. Data are shown as means ± standard deviation of two independent experiments. .

Table 1. Identification of Ginsenosides in Negative-ion and Positive-ion Mode by LC-PDA-MS/MS.

No.	Identfication	t_R (min)	$[M-H]^-$	$[M+HCOO]^-$	$[M+Na]^+$	Fragment ions in the negative mode	Fragment ions of $[M+Na]^+$ in the positive mode
1	ginsenoside Re	22.26		991.7	969.7	799.7 ($Y_{1\beta}$), 783.6 ($Y_{0\alpha}$), 765.8 ($Z_{0\alpha}$), 637.8 ($Y_{1\beta'}/Y_{0\beta}$), 619.8 ($Z_{1\beta}$), 475.4 ($Y_{0\beta'}$)	789.1, 643.7, 441.4, 423.5, 405.4, 349.4
2	ginsenoside Rg1	22.87*		845.7	823.7	637.7 ($Y_{0\alpha}/Y_{0\beta}$), 619.8 ($Z_{0\alpha}/Z_{0\beta}$), 475.6 ($Y_{0\alpha'}/Y_{0\beta'}$), 391.7 ($Y_{0\alpha'}$-$C_6H_{12}/Y_{0\beta'}$-C_6H_{12})	789.6, 643.6, 441.5, 423.5, 405.4
3	ginsenoside Rb1	29.95	1107.7	1153.8	1131.8	945.8 ($Y_{1\alpha}/Y_{1\beta}$), 783.7 ($Y_{0\alpha}/Y_{0\beta}$), 765.2 ($Z_{0\alpha}/Z_{0\beta}$), 621.9 ($Y_{1\alpha'}/Y_{1\beta'}$), 459.7 ($Y_{0\alpha'}/Y_{0\beta'}$)	789.5, 425.3, 407.4, 365.2
4	malonyl-Rb1	30.51	1193.9	1239.9	1217.9	1149.6 ($[M-H-COO]^-$), 1107.7 ($[M-H-malonyl]^-$), 1089.7 ($[M-H-malonyl-H_2O]^-$), 945.7 ($Y_{1\alpha}$), 927.8 ($Z_{1\alpha}$), 783.8 ($Y_{0\alpha}$), 765.7 ($Z_{0\alpha}$), 621.8 ($Y_{1\beta'}$), 459.5 ($Y_{0\beta'}$)	1173.7, 1101.7, 875.5, 831.1, 789.7, 451.3, 425.4, 407.4, 365.4, 335.3, 305.3
5	ginsenoside Rc	30.81	1077.8	1123.8	1101.8	945.7 ($Y_{1\alpha}$), 915.6 ($Y_{1\beta}$), 783.6 ($Y_{0\alpha}$), 765.5 ($Z_{0\alpha}$), 621.6 ($Y_{1\beta'}$), 603.8 ($Z_{1\beta'}$), 459.6 ($Y_{0\beta'}$)	875.5, 831.5, 789.6, 451.4, 425.4, 407.4, 335.3
6	ginsenoside Rb2	31.53	1077.7	1123.9	1101.6	945.8 ($Y_{1\alpha}$), 783.7 ($Y_{0\alpha}$), 765.7 ($Z_{0\alpha}$), 621.8 ($Y_{1\beta'}$), 603.6 ($Z_{1\beta'}$), 459.6 ($Y_{0\beta'}$)	875.6, 789.6, 451.6, 407.5, 335.4
7	ginsenoside Rb3	31.94	1077.8	1123.8	1101.8	945.7 ($Y_{1\alpha}$), 783.6 ($Y_{0\alpha}$), 765.8 ($Z_{0\alpha}$), 621.8 ($Y_{1\beta'}$), 459.7($Y_{0\beta'}$)	875.5, 831.6, 789.7, 627.6, 451.3, 425.5, 407.4, 365.3, 335.3
8	ginsenoside Ro	32.88	955.9		979.9	793.8 ($[M-H-glc]^-$), 631.8 ($[M-H-glc-glc]^-$), 569.5 ($[M-H-glc-glc-C_2H_6O_2]^-$), 475.8 ($[M-H-glc-glc-glc-acid]^-$)	845.6, 817.6, 641.4, 624.1, 439.4, 406.1, 393.4, 203.3
9	ginsenoside 20(*S*)-Rg2#	33.16	783.5	829.5	807.5	637.6 ($Y_{1\beta}$), 619.7 ($Z_{1\beta}$), 475.8 ($Y_{0\beta}$), 391.4 ($Y_{0\beta}$-C_6H_{12})	661.7, 441.6, 423.5, 405.6
10	quinquenoside R1	33.43	1149.8	1195.8	1173.8	1107.6 ($[M-H-acetyl]^-$), 945.8 ($Y_{1\alpha}$), 927.6 ($Z_{1\alpha}$), 783.4 ($Y_{0\alpha}$), 765.5 ($Z_{0\alpha}$), 621.9 ($Y_{1\beta'}$), 459.7 ($Y_{0\beta'}$)	831.5, 425.4, 407.4, 365.3

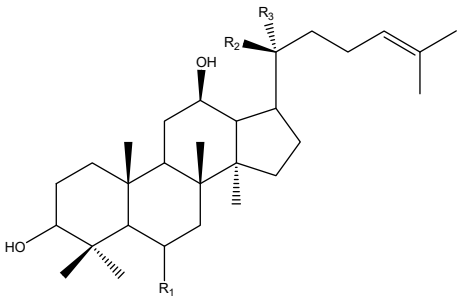
11	ginsenoside 20(*R*)-Rg2#	33.65*	783.6	829.6	807.6	637.7 ($Y_{1\beta}$), 475.8 ($Y_{0\beta}$), 391.4 ($Y_{0\beta}$-C_6H_{12})	661.6, 423.5, 405.4
12	ginsenoside Rh1#	34.84*		683.8	661.8	475.7 ($Y_{0\beta}$), 459.7 ($Z_{0\beta}$), 375.7 ($Z_{0\beta}$-C_6H_{12})	441.7, 423.5, 405.7
13	ginsenoside Rd	35.03	945.7	991.7	969.7	783.8 ($Y_{0\alpha}$/$Y_{1\beta}$), 765.7 ($Z_{0\alpha}$/$Z_{1\beta}$), 621.7 ($Y_{1\beta'}$/$Y_{0\beta}$), 603.5 ($Z_{0\beta}$), 459.7 ($Y_{0\beta'}$)	893.4, 875.5, 831.6, 789.6, 451.3, 425.4, 407.4
14	malonyl-Rd	35.74		1077.8	1055.8	987.6 ([M-H-COO]$^-$), 945.7 ([M-H-malonyl]$^-$), 783.7 ($Y_{0\alpha}$), 765.8 ($Z_{0\alpha}$), 621.6 ($Y_{1\beta'}$), 459.7 ($Y_{0\beta'}$)	1011.6, 893.5, 875.5, 831.6, 789.7, 451.3, 407.4
15	pseudoginsenoside Rc1	36.19*	987.5	1033.5	1011.6	945.7 ([M-H-acetyl]$^-$), 783.7 ($Y_{1\beta}$), 765.8 ($Z_{1\beta}$), 621.6 ($Y_{0\beta}$), 459.4 ($Y_{0\beta'}$)	969.4, 789.6, 627.8, 365.4
16	gypenoside-ⅩⅦ	36.46	945.7	991.7	969.7	783.7 ($Y_{1\alpha}$/$Y_{0\beta}$), 765.6 ($Z_{1\alpha}$/$Z_{0\beta}$), 621.7 ($Y_{1\alpha'}$/$Y_{0\alpha}$), 459.7 ($Y_{0\beta'}$)	627.6, 407.4, 365.2, 305.3,
17	malony-Rd isomer	37.21		1077.9	1055.8	987.5 ([M-H-COO]$^-$), 945.6 ([M-H-malonyl]$^-$), 783.8($Y_{0\beta}$), 765.5($Z_{0\beta}$), 621.7($Y_{1\alpha'}$), 459.7($Y_{0\alpha'}$)	1011.6, 831.6, 789.6, 713.5, 665.6, 627.6, 552.6, 451.3, 407.4, 365.1, 335.2
18	ginsenoside Rg6#	41.70*	765.8	811.8	789.8	619.7 ($Y_{1\beta}$), 601.7 ($Z_{1\beta}$), 459.7 ($Y_{0\beta}$), 375.7 ($Y_{0\beta}$-C_6H_{12})	679.8, 661.3, 643.8, 553.4, 453.4, 435.6
19	ginsenoside F2	42.01	783.8	829.8	807.8	619.7 ($Y_{0\alpha}$/$Y_{0\beta}$), 459.5 ($Y_{0\beta'}$), 375.7 ($Y_{0\beta'}$-C_6H_{12})	723.6, 664.6, 627.1, 519.6, 407.4, 365.4, 351.4, 337.4
20	ginsenoside F4#	42.48*	765.8	811.8	789.8	621.7 ($Y_{1\beta}$), 601.6 ($Z_{1\beta}$), 459.5 ($Y_{0\beta}$)	679.8, 661.9, 643.8, 553.4, 453.3, 435.7
21	ginsenoside 20(*S*)-Rg3#	45.28	783.8	829.8	807.8	621.7 ($Y_{1\beta}$), 459.5 ($Y_{0\beta}$), 375.8 ($Y_{0\beta}$-C_6H_{12})	645.8, 475.3, 457.8, 407.5, 365.7
22	ginsenoside 20(*R*)-Rg3#	45.94	783.8	829.8	807.8	621.6 ($Y_{1\beta}$), 459.6 ($Y_{0\beta}$)	645.8, 457.8, 407.5,
23	ginsenoside 20(*S*)-Rs3#	46.37*	825.8	871.7	849.7	783.4 ([M-H-acetyl]$^-$), 621.7 ($Y_{1\beta}$), 459.7 ($Y_{0\beta}$), 375.7 ($Y_{0\beta}$-C_6H_{12})	831.6, 813.6, 807.7, 789.7, 771.7, 645.6, 627.6, 451.5, 433.7, 407.6,

							389.6
24	ginsenoside 20(*R*)-Rs3#	46.78*	825.8	871.7	849.7	783.7 ([M-H-acetyl]$^-$), 621.5 ($Y_{1\beta}$), 459.6 ($Y_{0\beta}$)	813.7, 807.8, 789.7, 771.7, 645.6, 451.5, 433.7, 407.6
25	ginsenoside Rk1#	52.95	765.7	811.7	789.7	603.8 ($Y_{1\beta}$), 441.7 ($Y_{0\beta}$), 357.5 ($Y_{0\beta}$-C_6H_{12})	627.8, 365.7
26	Rs3-Δ20(21)H_2O#	53.93	807.8	853.7		765.7 ([M-H-acetyl]$^-$), 747.8 ([M-H-acetyl-H_2O]$^-$), 603.6 ($Y_{1\beta}$)	
27	ginsenoside Rg5#	54.53*	765.7	811.7	789.7	603.7 ($Y_{1\beta}$), 441.6 ($Y_{0\beta}$)	627.8, 475.6, 453.7, 435.6, 417.6, 365.7, 337.1,
28	Rs3-Δ20(22)H_2O#	54.87	807.7	853.8		765.7 ([M-H-acetyl]$^-$), 747.8 ([M-H-acetyl-H_2O]$^-$), 603.6 ($Y_{1\beta}$)	
29	ginsenoside Rh2#	57.81*	621.7	667.7	645.7	459.4 ($Y_{0\beta}$)	451.6, 407.6

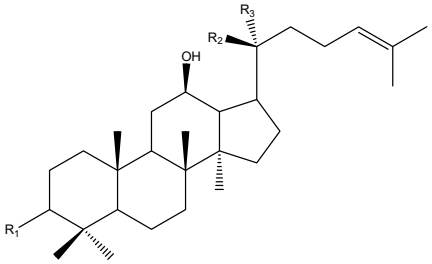
#, new ginsenosides from fermentation,

*retention time from extracted ion chromatograms (EICs)

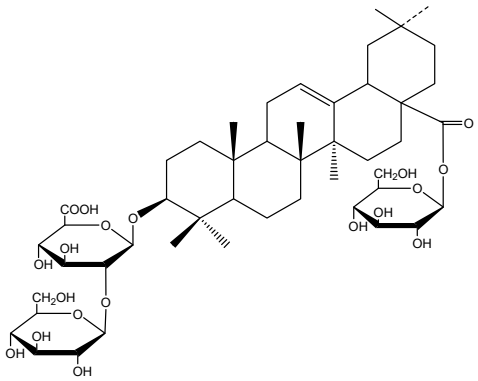
Figure 1



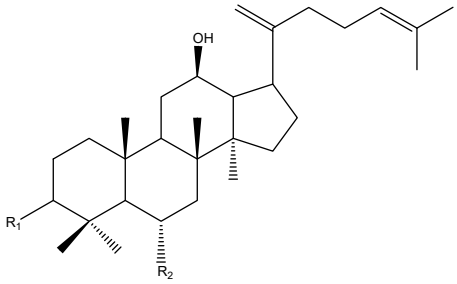
No.	Identification	Formula	R1	R2	R3
1	gisenoside Re	$C_{48}H_{82}O_{18}$	-Oglc(2-1)rha	-Oglc	-CH3
2	gisenoside Rg1	$C_{42}H_{72}O_{14}$	-Oglc	-Oglc	-CH3
9	20(*S*)-gisenoside Rg2	$C_{42}H_{72}O_{13}$	-Oglc(2-1)rha	-OH	-CH3
11	20(*R*)-gisenoside Rg2	$C_{42}H_{72}O_{13}$	-Oglc(2-1)rha	-CH3	-OH
12	20(*S*)-gisenoside Rh1	$C_{36}H_{62}O_{9}$	-Oglc	-OH	-CH3



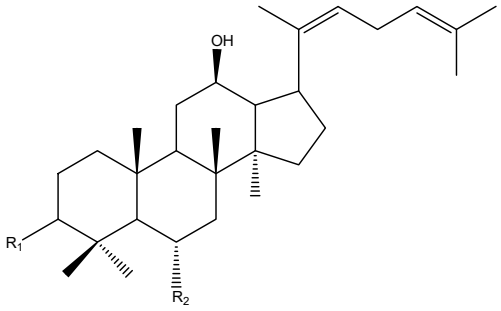
No.	Identification	Formula	R1	R2	R3
3	ginsenoside Rb1	$C_{54}H_{92}O_{23}$	-Oglc(2-1)glc	-Oglc(6-1)glc	-CH3
4	malonyl-Rb1	$C_{57}H_{94}O_{26}$	-Oglc(2-1)glc-malonyl	-Oglc(6-1)glc	-CH3
5	ginsenoside Rc	$C_{53}H_{90}O_{22}$	-Oglc(2-1)glc	-Oglc(6-1)araf	-CH3
6	ginsenoside Rb2	$C_{53}H_{90}O_{22}$	-Oglc(2-1)glc	-Oglc(6-1)arap	-CH3
7	ginsenoside Rb3	$C_{53}H_{90}O_{22}$	-Oglc(2-1)glc	-Oglc(6-1)xylp	-CH3
10	quinquenoside R1	$C_{56}H_{94}O_{24}$	-Oglc(2-1)glc-acetyl	-Oglc(6-1)glc	-CH3
13	ginsenoside Rd	$C_{48}H_{82}O_{18}$	-Oglc(2-1)glc	-Oglc	-CH3
14/17	malonyl-Rd/isomer	$C_{51}H_{84}O_{21}$	-Oglc(2-1)glc-malonyl	-Oglc	-CH3
15	pseudoginsenoside Rc1	$C_{50}H_{84}O_{19}$	-Oglc(2-1)glc-acetyl	-Oglc	-CH3
16	gypenoside ⅩⅦ	$C_{48}H_{82}O_{18}$	-Oglc	-Oglc(6-1)glc	-CH3
19	ginsenoside F2	$C_{42}H_{72}O_{13}$	-Oglc	-Oglc	-CH3
21	gisenoside 20(*S*)-Rg3	$C_{42}H_{72}O_{13}$	-Oglc(2-1)glc	-OH	-CH3
22	gisenoside 20(*R*)-Rg3	$C_{42}H_{72}O_{13}$	-Oglc(2-1)glc	-CH3	-OH
23	ginsenoside 20(*S*)-Rs3	$C_{44}H_{74}O_{14}$	-Oglc(2-1)glc-acetyl	-OH	-CH3
24	ginsenoside 20(*R*)-Rs3	$C_{44}H_{74}O_{14}$	-Oglc(2-1)glc-acetyl	-CH3	-OH
29	gisenoside 20(*S*)-Rh2	$C_{36}H_{62}O_{8}$	-Oglc	-OH	-CH3



No.	Identification	Formula
8	Ro	$C_{48}H_{76}O_{19}$



No.	Identification	Formula	R1	R2
18	ginsenoside Rg6	$C_{42}H_{70}O_{12}$	-OH	-Oglc(2-1)rha
25	ginsenoside Rk1	$C_{42}H_{70}O_{12}$	-Oglc(2-1)glc	-H
26	Rs3-Δ20(21)H2O	$C_{42}H_{68}O_{11}$	-Oglc(2-1)glc-acetyl	-H



No.	Identification	Formula	R1	R2
20	ginsenoside F4	$C_{42}H_{70}O_{12}$	-OH	-Oglc(2-1)rha
27	ginsenoside Rg5	$C_{42}H_{70}O_{12}$	-Oglc(2-1)glc	-H
28	Rs3-Δ20(22)H2O	$C_{42}H_{68}O_{11}$	-Oglc(2-1)glc-acetyl	-H

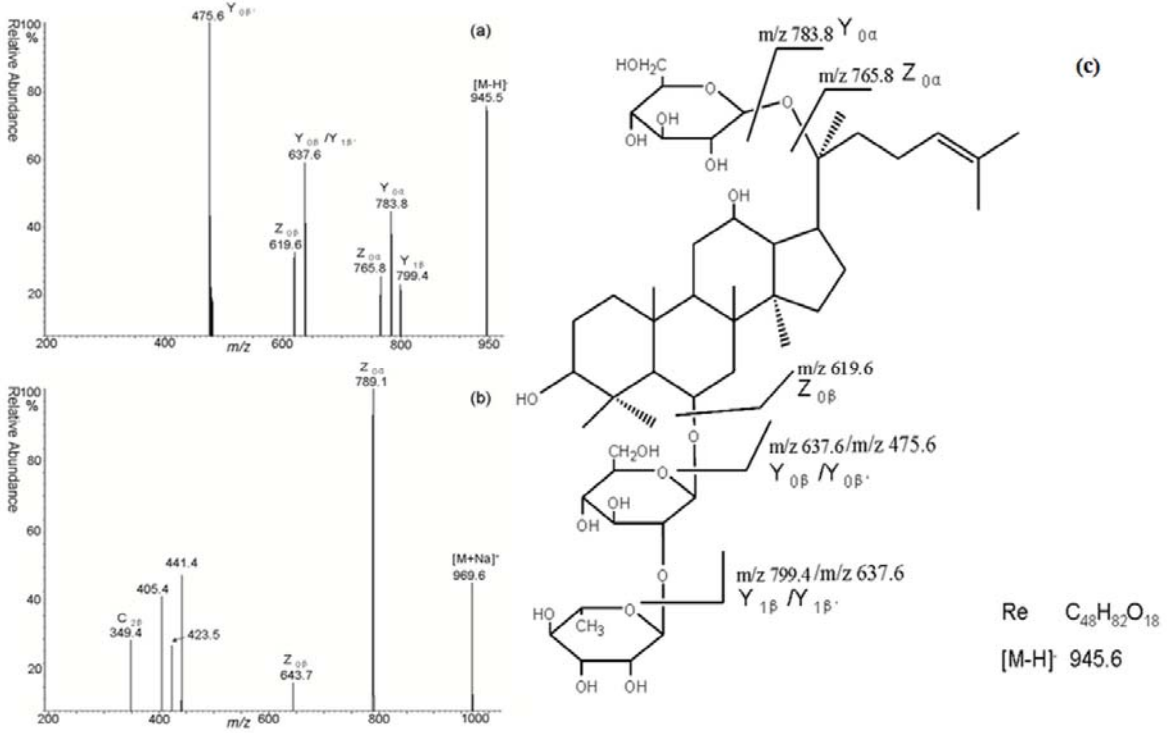

(a)
Relative Abundance
m/z
475.6
637.6
783.8
619.6
765.8
799.4
945.5
(b)
789.1
441.4
405.4
349.4
423.5
643.7
969.6
(c)
m/z 783.8 Y 0α
m/z 765.8 Z 0α
m/z 619.6 Z 0β
m/z 637.6/m/z 475.6 Y 0β /Y 0β'
m/z 799.4 /m/z 637.6 Y 1β /Y 1β'
Re C48H82O18
[M-H]- 945.6

Figure 2.

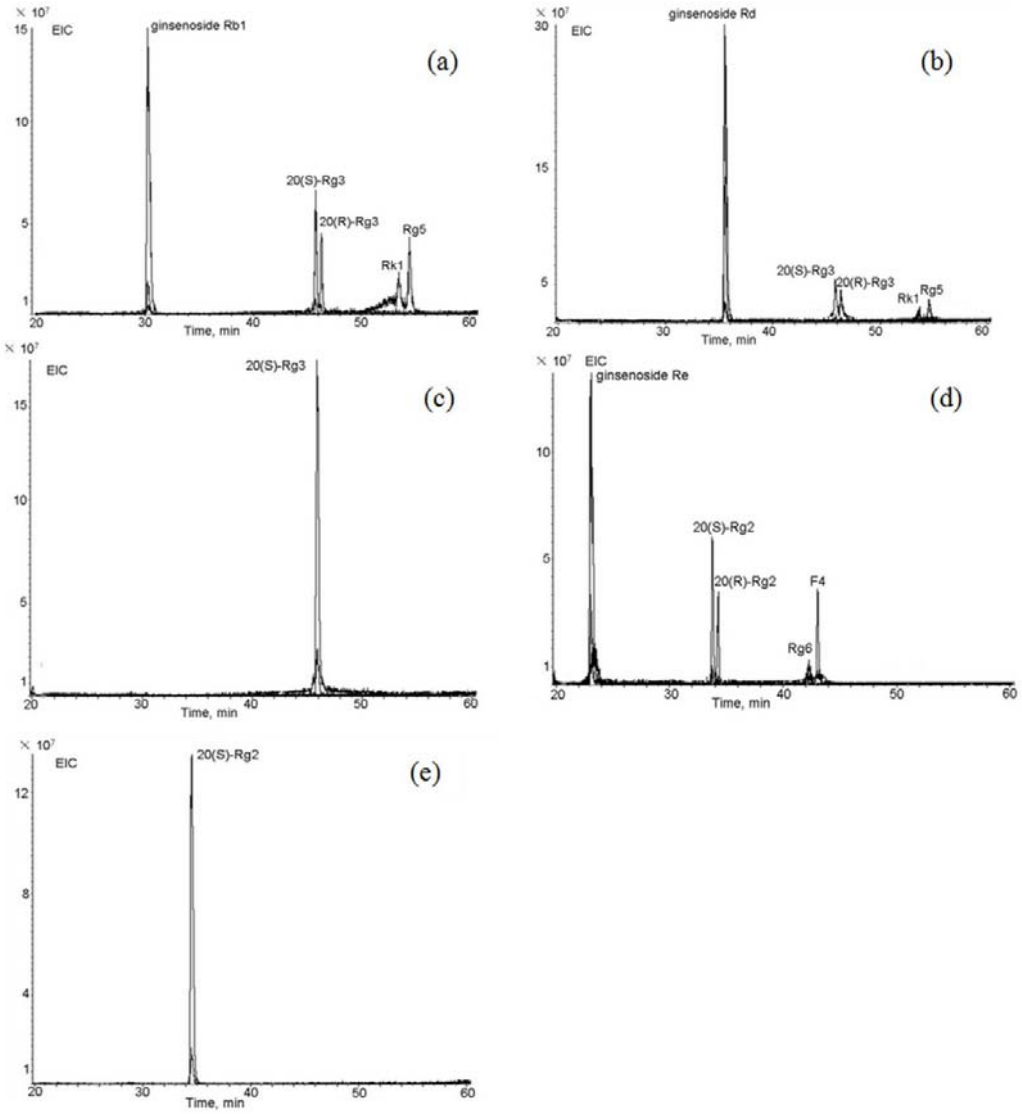

× 10^7
EIC
ginsenoside Rb1
(a)
20(S)-Rg3
20(R)-Rg3
Rg5
Rk1
Time, min
ginsenoside Rd
(b)
20(S)-Rg3
20(R)-Rg3
Rk1
Rg5
(c)
20(S)-Rg3
ginsenoside Re
(d)
20(S)-Rg2
20(R)-Rg2
F4
Rg6
(e)
20(S)-Rg2

Figure 3

Figure 4

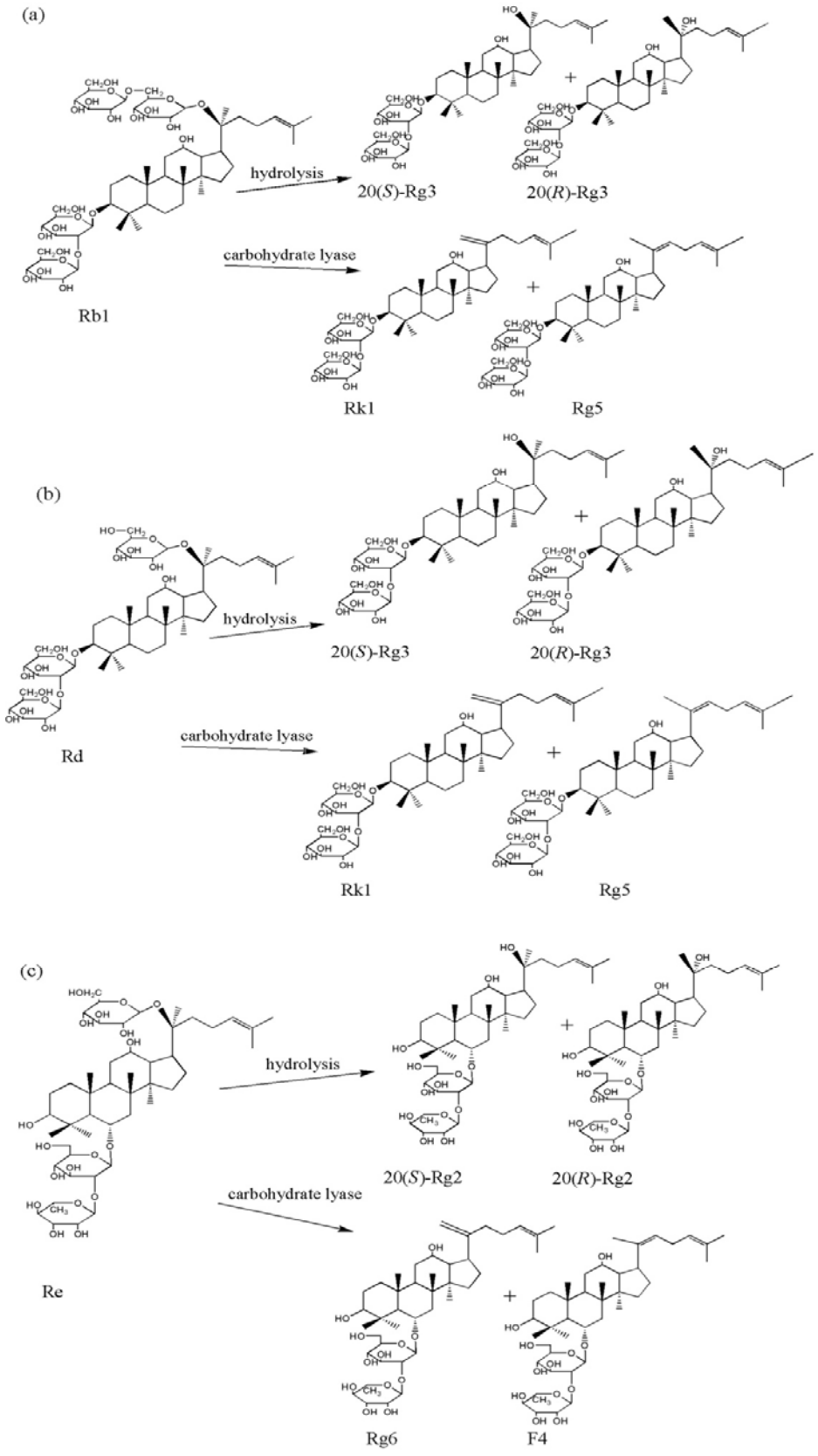
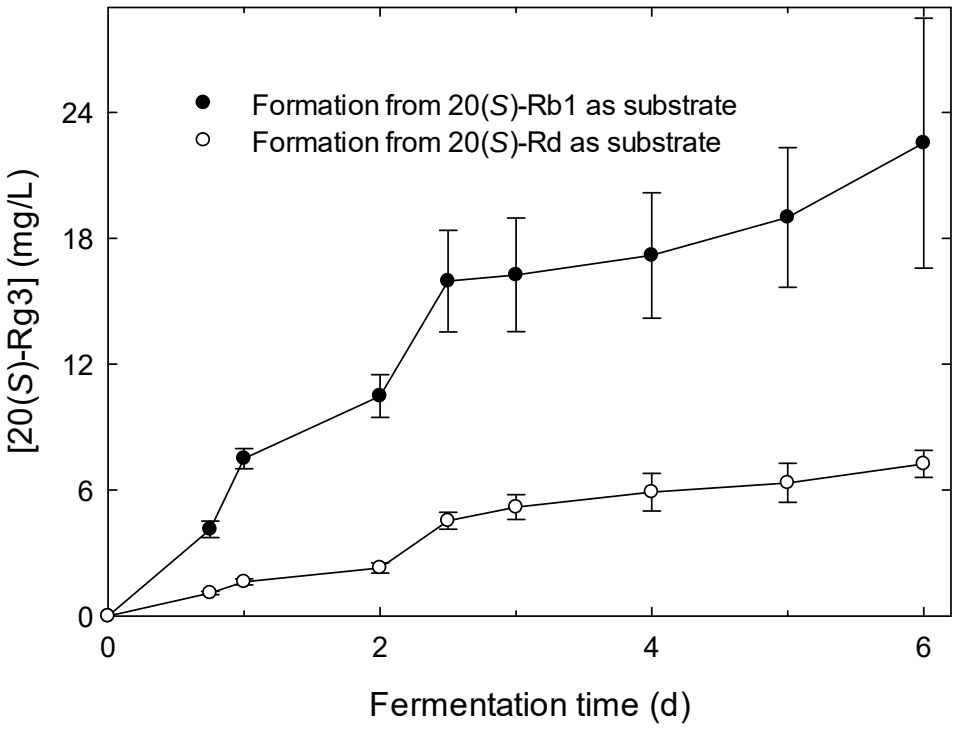

(a)
hydrolysis
20(S)-Rg3
20(R)-Rg3
carbohydrate lyase
Rb1
Rk1
Rg5
(b)
hydrolysis
20(S)-Rg3
20(R)-Rg3
carbohydrate lyase
Rd
Rk1
Rg5
(c)
hydrolysis
20(S)-Rg2
20(R)-Rg2
carbohydrate lyase
Re
Rg6
F4

Figure 5


Formation from 20(S)-Rb1 as substrate
Formation from 20(S)-Rd as substrate
[20(S)-Rg3] (mg/L)
Fermentation time (d)
0
6
12
18
24
0
2
4
6