J Ind Microbiol Biotechnol (2012) 39:521–527
DOI 10.1007/s10295-011-1058-9

ORIGINAL PAPER

Co-transformation of *Panax* major ginsenosides Rb_1 and Rg_1 to minor ginsenosides C–K and F_1 by *Cladosporium cladosporioides*

Lunpeng Wu · Yan Jin · Chengri Yin · Longlv Bai

Received: 17 March 2011 / Accepted: 8 November 2011 / Published online: 20 January 2012

Abstract Rb_1 and Rg_1 are the major ginsenosides in protopanaxadiol and protopanaxatriol. Their content in ginsenosides was 23.8 and 17.6%, respectively. A total of 22 isolates of β-glucosidase producing microorganisms were isolated from the soil of a ginseng field using Esculin-R2A agar. Among these isolates, the strain GH21 showed the strongest activities to convert ginsenoside Rb_1 and Rg_1 to minor ginsenosides compound-K and F_1, respectively. Ginsenosides Rb_1 and Rg_1 bioconversion rates were 74.2 and 89.3%, respectively. Meanwhile, the results demonstrated that the ginsenoside Rg_1 could change the biotransformation pathway of ginsenoside Rb_1 by inhibiting the formation of the intermediate metabolite gypenoside-XVII. GH21 was identified as a *Cladosporium cladosporioides* species based on the internal transcribed spacers (ITS) ITS1-5.8S-ITS2 rRNA gene sequences constructed phylogenetic trees.

Keywords Biotransformation · Co-transformation · Ginsenoside Rb_1 · Ginsenoside Rg_1 · Minor ginsenoside

Introduction

Ginseng, the root of *Panax ginseng* C. A. Meyer, Araliaceae, is a well-known medicinal plant. It has been used as a traditional natural medicine in China, Korea, and Japan for thousands of years. Its chemical properties and pharmaceutical functions have been intensively studied throughout the world. Ginsenosides are regarded as the principal components responsible for numerous pharmacological properties. Up to now, more than 40 ginsenosides have been isolated and characterized from ginseng roots, which including with the major ginsenosides Rb_1, Rb_2, Rc, Rg_1 and Re that constituting more than 90% of the total ginsenosides [18]. Ginsenosides Rb_1 and Rg_1 have a higher ginseng content. Ginsenoside Rb_1 is the major ingredient of the protopanaxadiol group saponins. Ginsenoside Rg_1 is one of the major protopanaxatriol group saponins, which is the most abundant in all the ginsenosides. The content of ginsenoside Rb_1 and ginsenoside Rg_1 in total ginsenosides was 23.8 and 17.6% [19], respectively. Ginsenoside was reported to show various pharmacological activities such as protection from free-radical damage, maintaining normal cholesterol and blood pressure, and rescuing hippocampal neurons from lethal ischemic damage [16, 20]. In addition, it was reported to show the activities of stimulating central nervous, improving learning and memory, immunoregulatory action, and anti-fatigue [22, 23].

In recent decades, many studies have focused on the pharmacological activities of the minor ginsenosides, as their activities were found to be better than those of the major ginsenosides. The minor ginsenoside compound K has reported to show significant biological activities, such as antigenotoxic activity, antiallergic effect, and the prevention of tumor invasion and metastasis [10, 15]. In addition, Compound K shows potential hepatoprotective and anti-inflammatory activities [6, 14]. Ginsenoside F_1 is an enzymatic metabolite generated from ginsenoside Rg_1, which shows anti-proliferation, anti-migration, and keratinocytes protective effects [13, 21]. Therefore, many studies

L. Wu · Y. Jin · C. Yin (✉)
Key Laboratory of Natural Resources of Changbai Mountain and Functional Molecules, Ministry of Education, Yan Bian University, Gongyuan Road, Yanji 133002, Jilin, China
e-mail: yin_cheng_ri@hotmail.com

L. Wu · L. Bai
National Ginseng Products Quality Supervision Inspection Center, Yan ji 133000, Jilin, China

have aimed to convert major ginsenosides to the more active minor ginsenosides [1, 4, 9]. Although the production of minor ginsenoside has been performed using ginsenoside Rb_1 or Rg_1 in microorganisms, Rb_1 and Rg_1 co-transformation has not yet been reported [2, 5, 7, 11].

In this study, we isolated the β-glucosidase-producing microorganisms from the soil of a ginseng field using Esculin-R2A agar and investigated the activity co-transforming ginsenosides Rb_1 and Rg_1 to minor ginsenosides compound-K (C–K) and F_1.

Materials and methods

Materials

Standard ginsenosides including 20(S)-Rb_1, 20(S)-Rd, 20(S)-Rg_1, F_2, F_1, and C–K were obtained from the Chengdu Mansite Pharmaceutical Co., Ltd., China. R2A agar was purchased from Difco. Silica gel-60 used for TLC was purchased from Merck KgaA, Darmstadt, Germany. All chemicals and solvents were of analytical or HPLC grade.

Screening of microorganisms producing β-glucosidase

Esculin-R2A agar was used to isolate β-glucosidase-producing microorganisms. Esculin-R2A agar contains (per 1 l): esculin 1 g and ferric citrate 0.5 g with 15.2 g R2A agar, and is autoclaved at 121°C for 15 min. The microorganisms producing β-glucosidase and that hydrolyze esculin appeared as colonies surrounded by a reddish-brown to dark brown zone.

The microorganisms were isolated from the soil of a ginseng field (Changbai Mountain, China) by direct plating onto Esculin-R2A agar. Single colonies from these plates were purified by transferring onto new plates. Pure cultures were checked for shape, color, and size of colonies.

Biotransformation of ginsenosides

The biotransformation procedure was carried out in 100-ml Erlenmeyer flasks containing 40 ml biotransformation medium and 0.4 mg l^{-1} Rb_1 or Rg_1 as the carbon source in a shaking incubator (150 rpm) at 30°C. The biotransformation medium consists of 0.5 g NH_4Cl, 1.0 g K_2HPO_4, 0.5 g KH_2PO_4, 0.25 g $MgSO_4$ and 1.0 g yeast extract l^{-1}. All medium was sterilized at 121°C for 15 min and the initial pH was 7.0. The reaction mixture was extracted with *n*-butanol saturated with H_2O and analyzed by TLC and HPLC.

Analytical methods

TLC analysis was carried out using a Silica Gel 60 plates and a solvent system of $CHCl_3$-CH_3OH-H_2O (10:5:1 v/v/v) as the developing solvent. The spots on the TLC plates were detected by spraying 10% (v/v) H_2SO_4 (in ethanol) followed by heating at 110°C for 10 min.

The reaction mixture was extracted with *n*-butanol saturated with H_2O, evaporated in vacuo, and the residue was dissolved in CH_3OH and applied to the HPLC analysis, HPLC used a C_{18} column (250 × 4.6 mm, ID 5 μm) with H_2O (solvent A) and acetonitrile (solvent B) at A/B ratios of 75/25, 68/32, 45/55, 40/60, 0/100, 0/100, 75/25, and 75/25, with run times of 0, 10, 15, 20, 25, 27, 40, and 50 min, respectively, at a flow rate of 1 ml min^{-1}. Detection wavelength was 203 nm.

Bioconversion rates of ginsenoside C–K and F_1 were calculated as follows:

$$\text{Bioconversion rate of ginsenoside C} - \text{K}\,(\%) = \frac{\text{Weight of C} - \text{K}/\text{MW of C} - \text{K}}{\text{Weight of Rb}_1\ /\ \text{MW of Rb}_1}$$

$$\text{Bioconversion rate of ginsenoside F1}\,(\%) = \frac{\text{Weight of F}_1/\text{MW of F}_1}{\text{Weight of Rg}_1\ /\ \text{MW of Rg}_1}$$

where MW is the molecular weight, i.e., MW of Rb_1 is 1,108, MW of C–K is 622, MW of Rg_1 is 801, and MW of F_1 is 639. All the experiments were conducted in triplicate and the results are expressed as mean values ± SD.

Molecular methods

The ITS rDNA gene sequences of the strain GH21 was sequenced by the Shanghai Majorbio Bio-Pharm Technology Co. Ltd., China. The ITS rDNA gene sequences of the related taxa were obtained from GenBank. The phylogenetic tree was constructed using the neighbor-jointing method through the MEGA 4.1 program. A bootstrap analysis with 1,000 replicates was also conducted to obtain confidence levels for the branches. The closest type strains were included in the phylogenetic trees.

Results

Initial screening of β-glucosidase-producing microorganisms

Twenty-two microorganisms were initially screened using the Esculin-R2A agar, for production of β-glucosidase. The microorganisms producing β-glucosidase hydrolyze esculin appeared as colonies surrounded by a reddish-brown to dark brown zone, which showed β-glucosidase activity. The black colonies were picked and transferred to the fresh Esculin-R2A agar. The morphological characteristics of pure cultures were checked for size, shape, and color of colonies.

Screening of Co-transformation microorganisms

All the β-glucosidase-producing microorganisms were assayed to verify their activity for converting ginsenoside Rb_1 or Rg_1, respectively. Microorganisms that were able to transform both ginsenosides Rb_1 and Rg_1 were selected to co-transform the mixture of ginsenosides Rb_1 and Rg_1. Among the 22 β-glucosidase-producing strains, GH9, GH21, and GH26 were shown to be able to convert ginsenoside Rb_1 to less polar metabolite by TLC, with an Rf value similar to ginsenoside C–K. Among these isolates, only GH21 shows the most potent ability to convert ginsenoside Rg_1.

Co-transformation of ginsenosides Rb_1 and Rg_1 by strain GH21 and TLC assay

The TLC results are shown in Fig. 1a, along with the concentrations of ginsenoside Rb_1 and the decomposition products Rd, gypenoside-XVII, F_2 exhibited regular changes with reaction time. Ginsenoside Rb_1 was converted into gypenoside-XVII and Rd by hydrolysis of a glucose unit at the C-3 position or C-20 position of the ginsenoside aglycone. Ginsenoside F_2 was produced from gypenoside-XVII and Rd by additional hydrolysis of a single glucose moiety. The concentration of F_2 reached the highest level after 4 days and then gradually decreased. However, the concentration of C–K increased continuously from 4 to 7 days. This indicated that the ginsenosides gypenoside-XVII, Rd, and F_2 were intermediate metabolites, and C–K was the final product. As shown in Fig. 1b, ginsenoside Rg_1 was transformed to F_1 as the sole metabolite after 7 days of incubation. The TLC results of the co-transformation of ginsenosides Rb_1 and Rg_1 are presented in Fig. 1c. Ginsenoside Rb_1 was metabolized to Rd, F_2, and C–K but not generate intermediate metabolite gypenoside-XVII during the entire reaction time. In addition, ginsenoside Rg_1 was converted to F_1 by the strain GH21.

Co-transformation of ginsenosides Rb_1 and Rg_1 by strain GH21 and HPLC assay

The HPLC profiles of the reaction mixture of ginsenosides Rb_1 and Rg_1 in strain GH21 after 7 days incubation are shown in Fig. 2.

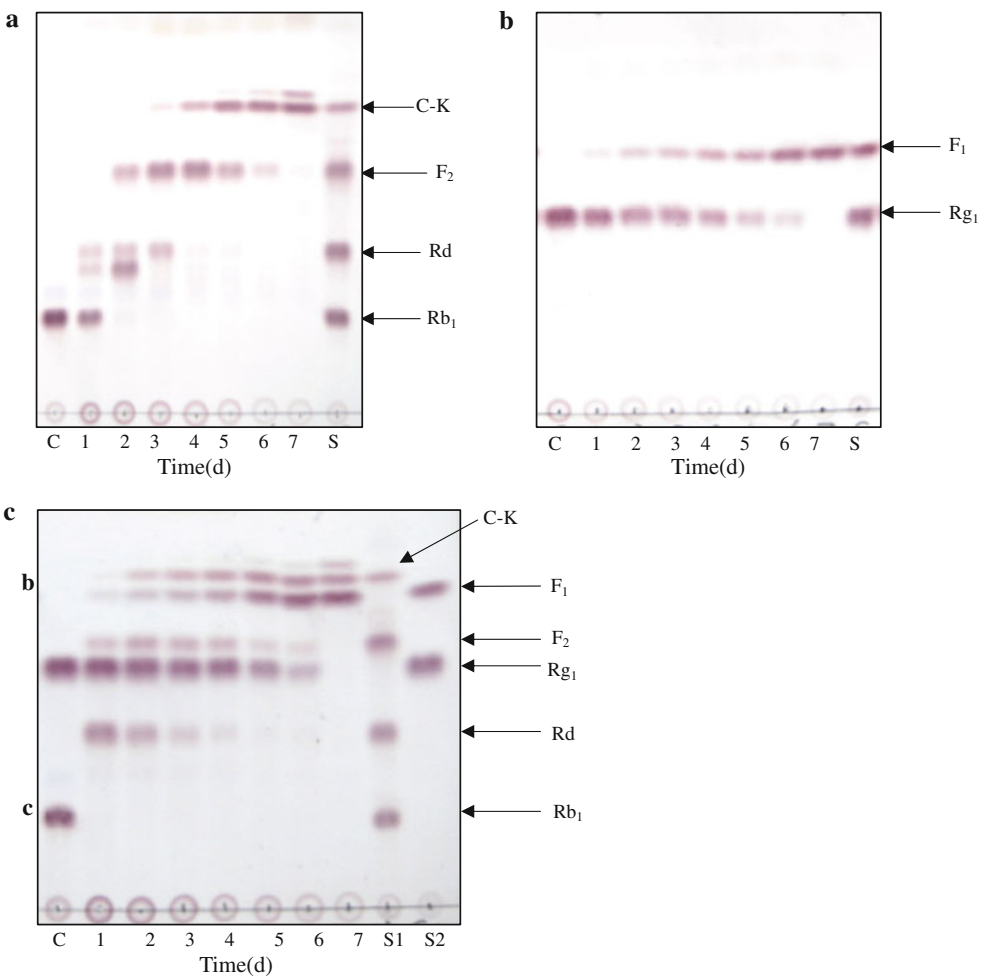

Fig. 1 Time-course TLC analysis of metabolites of ginsenoside Rb_1 converted by strain GH21. **a** Ginsenoside Rb_1 was used as substrate. **b** Ginsenoside Rg_1 was used as substrate. **c** Ginsenoside $Rb_1 + Rg_1$ were used as substrate. *C* Control; *S1* and *S2* saponin standards

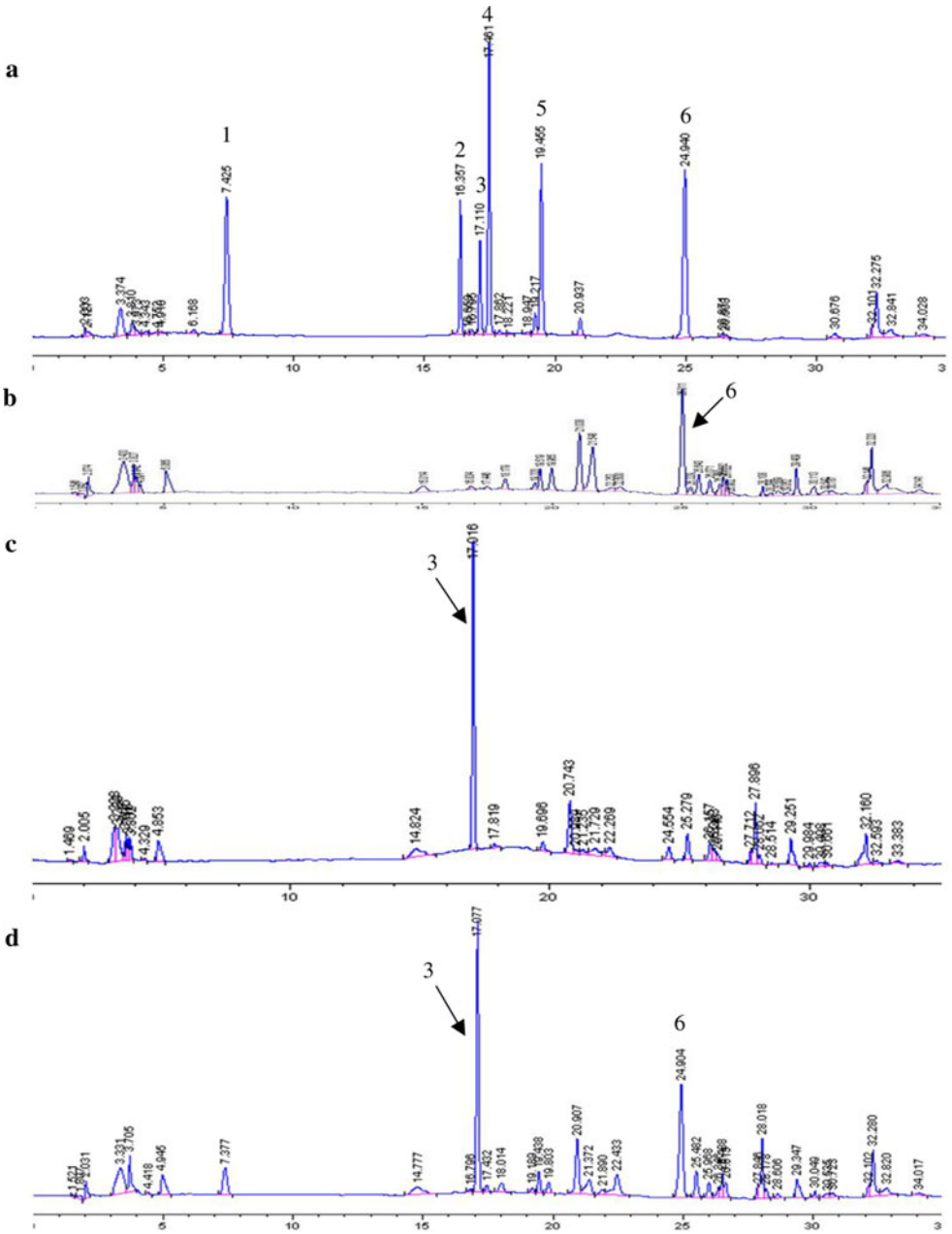

Fig. 2 HPLC profiles of metabolites of ginsenoside Rb_1 and Rg_1 converted by GH21. **a** Ginsenoside standards. **b** Ginsenoside Rb_1 metabolites. **c** Ginsenoside Rg_1 metabolite. **d** Ginsenoside Rb_1 and Rg_1 co-transformation metabolites

The peaks with retention time 7.43, 16.36, 17.11, 17.48, 19.46, and 24.94 min correspond to ginsenosides Rg_1, Rb_1, F_1, Rd, F_2, and C–K, respectively. As shown in Fig. 2b, the peak for ginsenoside Rb_1 disappeared after 7 days and then a new peak appeared. It had a retention time consistent with that of C–K. As shown Fig. 2c, ginsenoside Rg_1 entirely disappeared, and then followed by the appearance of a new peak. Its retention time was similar to that of ginsenoside F_1. Figure 2d shows the ginsenosides Rb_1 and Rg_1 co-transformation metabolites after 7 days. Two peaks indicate that a significant amount of C–K and F_1 were detected as the final metabolites. Ginsenosides Rb_1 and Rg_1 bioconversion rates were 74.2 and 89.3%, respectively.

Phylogenetic study

Internal transcribed spacers ITS1-5.8S-ITS2 rRNA gene sequences of the strain GH21 were aligned with those of the type strains found to have the closest taxonomic relationships. The phylogenetic tree is shown in Fig. 3. The ITS rRNA gene sequences of the related taxa were obtained from GenBank. Strain GH21 was calculated to belong to the *Cladosporium*, with the highest degree of ITS rRNA gene sequences similarity found with the *Cladosporium cladosporioides* strain STE-U 3683 (AY251074) (100%), *Cladosporium cladosporioides* strain ATCC 201098 (AF393688) (99%), and *Cladosporium cladosporioides* strain ATCC 200941 (AF393691)

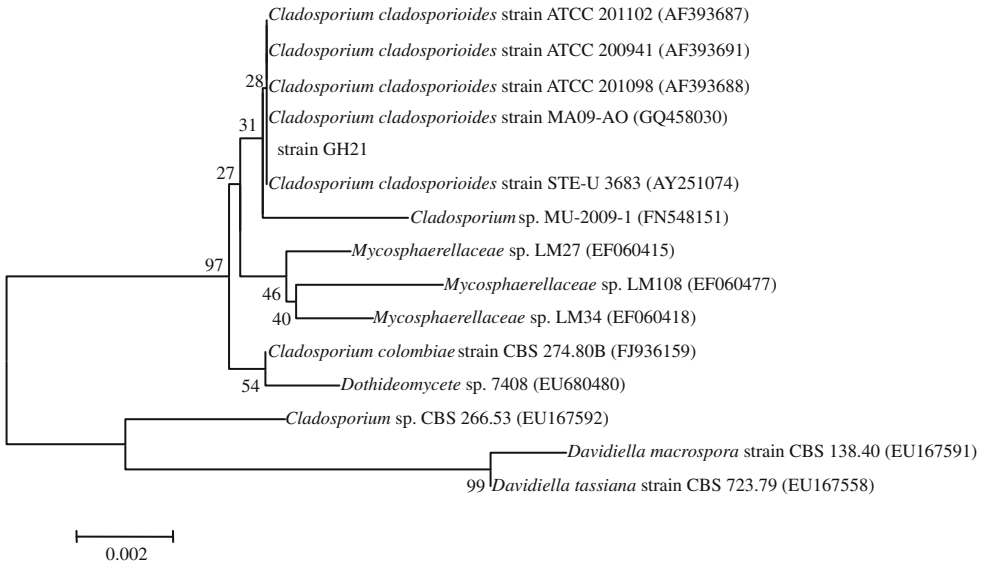

Fig. 3 Phylogenetic trees based on the ITS rRNA gene sequences, showing the phylogenetic relationships of the strain GH21

(99%). Therefore, the strain GH21 is classified as a *Cladosporium cladosporioides*. As determined by taxonomic evaluation, the strain showing less than 1% difference in its ITS rRNA gene sequence with the corresponding type stains were assumed to belong to the same species as the type stains.

Discussion

Ginsenoside compound K and ginsenoside F_1 are promising natural product that could be used for the treatment of numerous human diseases. Unfortunately, the methods currently available for the commercial production of ginsenoside compound K and F_1 are difficult. It thus limited the availability and development of these compounds. There are reports on microbial sources able to convert the major ginsenoside Rb_1 or ginsenoside Rg_1 to minor ginsenosides usually through the pathway of $Rb_1 \rightarrow Rd \rightarrow F_2 \rightarrow$ C–K by microorganisms; Other pathways for C–K production such as $Rb_1 \rightarrow$ gypenoside XVII $\rightarrow$ gypenoside LXXV $\rightarrow$ C–K have been reported [3, 12], The main transformation pathway of ginsenoside Rg_1 are supposed to be as follows: $Rg_1 \rightarrow Rh_1$ or $F_1 \rightarrow$ PPT (M_4) have been reported previously [8, 17]. However, most of them have lack of specificity.

In this study, it is the first report on *Cladosporium cladosporioides* species microorganism co-transformation of major ginsenoside Rb_1 and Rg_1 to the minor ginsenoside C–K and F_1. When ginsenoside Rb_1 was experimented as substrate of strain GH21, it was converted into C–K through two different transformation pathways, $Rb_1 \rightarrow$ Rd or gypenoside XVII $\rightarrow F_2 \rightarrow$ C–K, as shown in the pathway in Fig. 4a. When ginsenoside Rg_1 was experimented as substrate of strain GH21, it was converted into F_1 through transformation pathway $Rg_1 \rightarrow F_1$, as shown in the pathway in Fig. 4b. When compared to the previous reports, the strain GH21 was found high selectivity in cleaving the glucosidic linkage at the C-6 position of ginsenoside Rg_1, without attacking the glucosidic linkage at the C-20 position. In the throughout transformation period, none of Rh_1 and PPT were generated. This high selectivity is suitable for industrial production.

From the above-mentioned results, ginsenoside Rg_1 can change the biotransformation pathway of ginsenoside Rb_1 when ginsenosides Rb_1 and Rg_1 were co-transformed. Ginsenoside Rb_1 was inhibited in being transformed to the intermediate metabolites gypenoside-XVII. This suggested that Rb_1 was converted by different enzymes secreted by the strain GH21. In the course of co-transformation, ginsenoside Rg_1 restrain the activity of enzyme by hydrolyses of a terminal glucose moiety at C-3 position. Taking these data into account, we propose the biotransformation pathway of ginsenosides Rb_1 and Rg_1 by the strain GH21 as shown in Fig. 4c, this deduction is consistent with the spectrum profiles obtained from TLC and HPLC. Further work has been undertaken to isolate and purify the enzymes so that the production cycle can be shortened and the yield can be increased.

Conclusions

In the course of co-transformation, *Cladosporium cladosporioides* species transformed ginsenoside Rb_1 and Rg_1 with high bioconversion rate of 74.2 and 89.3%, respectively. We found that the ginsenoside Rg_1 could change the biotransformation pathway of ginsenoside Rb_1 by

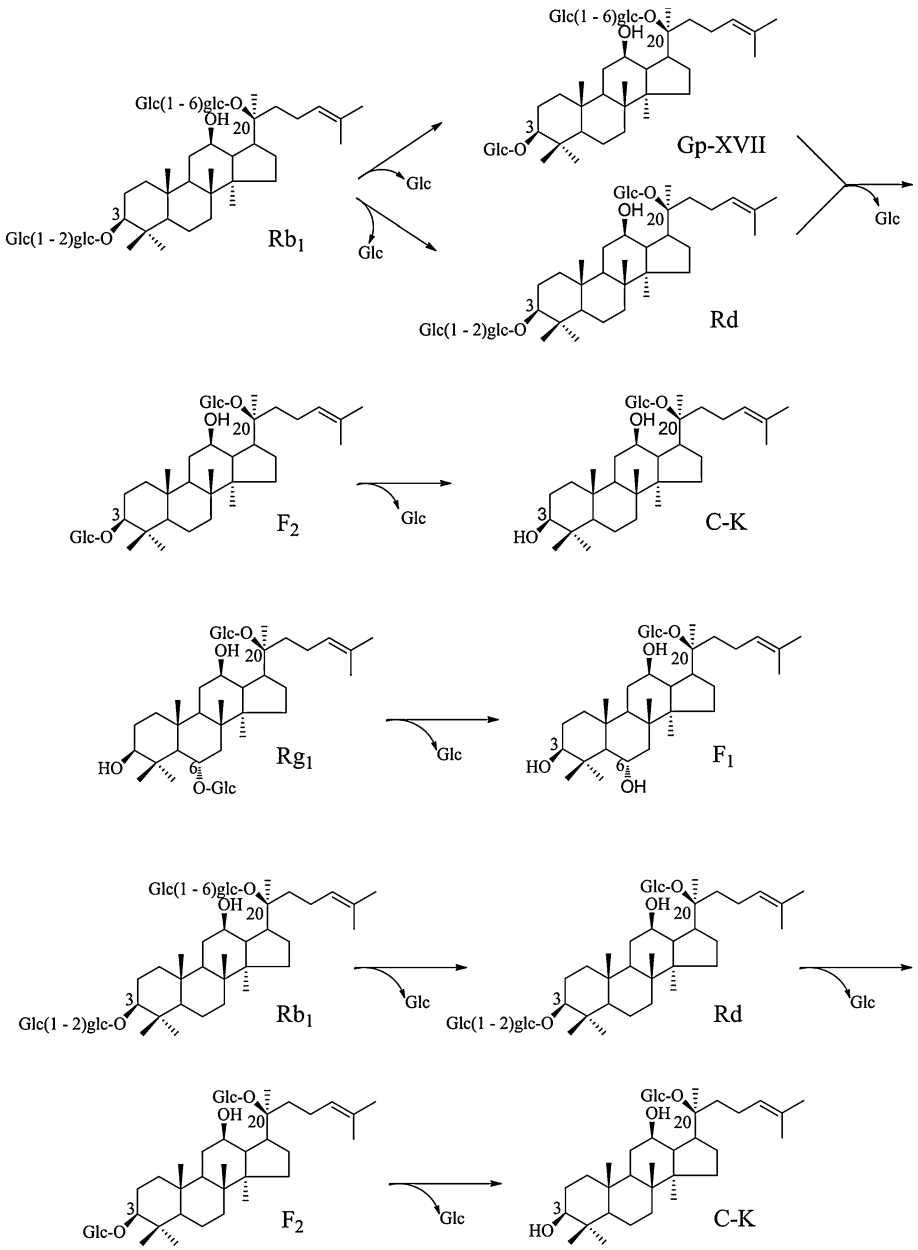

Fig. 4 A proposed biotransformation pathway of ginsenoside Rb_1, Rg_1, and $Rb_1 + Rg_1$ by GH21. **a** Ginsenoside Rb_1; **b** ginsenoside Rg_1; **c** ginsenoside $Rb_1 + Rg_1$

inhibiting the formation of the intermediate metabolite gypenoside-XVII.

Acknowledgments This work was supported by the National Natural Science Foundation of China (No.20862017) and National Ginseng Products Quality Supervision Inspection Center.

References

1. Chen GT, Yang M, Song Y, Lu ZQ, Zhang JQ, Huang HL, Wu LJ, Guo DA (2008) Microbial transformation of ginsenoside Rb_1 by *Acremonium strictum*. Appl Microbiol Biotechnol 77:1345–1350
2. Cheng LQ, Kim MK, Lee JW, Lee YJ, Yang DC (2006) Conversion of major ginsenoside Rb_1 to ginsenoside F_2 by *Caulobacter leidyia*. Biotechnol Lett 28:1121–1127
3. Cheng LQ, Na JR, Kim MK, Bang MH, Yang DC (2007) Microbial conversion of minor ginsenoside F_2 and gypenoside XVII by *intrasporangium* sp.GS603 isolated from soil. J Microbiol Biotechnol 17:1937–1943
4. Chi H, Kim DH, Ji GE (2005) Transformation of ginsenosides Rb_2 and Rc from *Panax ginseng* by food microorganisms. Biol Pharm Bull 28:2102–2105
5. Chi H, Ji GE (2005) Transformation of ginsenosides Rb_1 and Re from Panax ginseng by food microorganisms. Biotechnol Lett 27:765–771
6. Choi K, Kim M, Ryu J, Choi C (2007) Ginsenosides compound K and Rh2 inhibit tumor necrosis factor-α-induced activation of the

NF-kB and JNK pathways in human astroglial cells. Neurosci Lett 421:37–41
7. Dong A, Ye M, Guo H, Zheng J, Guo D (2003) Microtransformation of ginsenoside Rb_1 by *Rhizopus stolon* and *Curvularia lunata*. Biotechnol Lett 25:339–344
8. Dong AL, Cui YJ, Guo H, Zheng JH, Guo D (2001) Microbiological transformation of ginsenoside Rg1. J Chin Pharm Sci 10:115–118
9. Han Y, Sun BS, Hu XM, Zhang H, Jiang BH, Spranger MI, Zhao YQ (2007) Transformation of bioactive compounds by *Fusariun sacchari* fungus isolated from the soil-cultivated Ginseng. J Agric Food Chem 55:9373–9379
10. Hasegawa H (2004) Proof of the mysterious efficacy of ginseng: Basic and clinical trials: metabolic activation of ginsenoside: deglycosylation by intestinal bacteria and esterification with fatty acid. J Pharmacol Sci 95:153–157
11. Kim MK, Lee JW, Lee KY, Yang DC (2005) Microbial conversion of major ginsenoside Rb_1 to pharmaceutically active minor ginsenoside Rd. J Microbiol 43:456–462
12. Lee BH, You HJ, Park MS, Kwin B, Ji GE (2006) Transformation of the glycosides from food materials by probiotics and food microorganisms. J Microbiol Biotechnol 16:497–504
13. Lee EH, Cho SY, Kim SJ, Shin ES, Chang HK et al (2003) Ginsenoside F_1 protects human HaCaT keratinocytes from ultraviolet-B-induced apoptosis by maintaining constant levels of Bcl-2. J Invest Dermatol 121:607–613
14. Lee HU, Bae EA, Han MJ, Kim NJ, Kim DH (2005) Hepatoprotective effect of ginsenoside Rb_1 and compound K on tert-butyl hydroperoxide-induced liver injury. Liver Int 25:1069–1073
15. Lee SJ, Sung JH, Lee BH (1999) Antitumor activity of a novel ginseng saponin metabolite in human pulmonary adenocarcinoma cells resistant to cisplatin. Cancer Lett 144:39–43
16. Lim JH, Wen TC, Matsuda S, Tanaka J, Maeda N, Peng H, Aburaya J, Ishihara K, Sakanaka M (1997) Protection of ischemic hippocampal neurons by ginsenoside Rb_1, a main ingredient of ginseng root. Neurosci Res 28:191–200
17. Odani T, Tanizawa H, Takino Y (1983) Studies on the absorption, distribution, excretion and metabolism of ginseng saponins, the absorption, distribution and excretion of ginsenoside Rg_1 in the rat. Chem Pharm Bull 31:1059–1066
18. Park JH (2004) Sun a new processed ginseng with fortified activity. Food Ind Nutr 9:23–27
19. Son JW, Kim HJ, Oh DK (2008) Ginsenoside Rd production from the major ginsenoside Rb_1 by beta-glucosidase from *Thermus caldophilus*. Biotechnol Lett 30:713–716
20. Wang XY, Zhang JT (2003) Effect of ginsenoside Rb_1 on long-term potentiation in the dentate gyrus of anaesthetized rats. J Asian Nat Prod Res 5:1–4
21. Yoo DS, Rho HS, Lee YG, Yeom MH et al (2011) Ginsenoside F_1 modulates cellular responses of skin melanoma cells. J Ginseng Res 35:86–91
22. Zhang JT, Liu Y, Qu ZW (1988) Influence of ginsenoside Rb_1 and Rg_1 on some central neurotransmitter receptors and protein biosynthesis in the mouse brain. Acta Pharm Sin 23:12–16
23. Zhang DS, Zhang JT (2000) Effects of total ginsenoside on learning and memory impairment induced by transient betaamyloid peptide. Chin Pharm Bull 16:422–425