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Highly Regioselective Biotransformation of Protopanaxadiol-type and Protopanaxatriol-type Ginsenosides in the Underground Parts of *Panax notoginseng* to 18 Minor Ginsenosides by *Talaromyces flavus*

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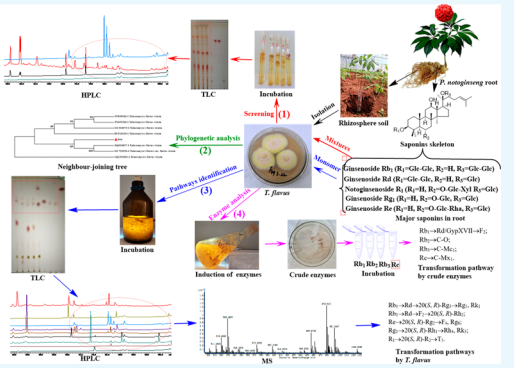
ABSTRACT: The transformation of major ginsenosides to minor ginsenosides by microorganisms was considered to be an environmentally friendly method. Compared with GRAS (generally recognized as safe) strains, non-food-grade microorganisms could transform polar ginsenosides to various minor ginsenosides. In this study, *Talaromyces flavus* screened from the *P. notoginseng* rhizosphere was capable of transforming PPD-type and PPT-type ginsenosides in the underground parts of *P. notoginseng* to 18 minor ginsenosides. The transformation reactions invovled deglycosylation, epimerization, and dehydration. To the best of our knowledge, this transformation characteristic of *T. flavus* was first reported in fungi. Its crude enzyme can efficiently hydrolyze the outer glucose linked to C-20 and C-3 in major ginsenosides Rb_1, Rb_2, Rb_3, Rc, Rd, and 20(*S*)-Rg_3 within 48 h. The transformation of major ginsenosides to minor ginsenosides by *T. flavus* will help raise the functional and economic value of *P. notoginseng*.



INTRODUCTION

Panax notoginseng (Burk.) F. H. Chen has long been used as a medicine[1] and functional food.[2,3] Ginsenosides were major bioactive constituents of *P. notoginseng*.[4] Underground parts of *P. notoginseng* were the frequently used extraction part of ginsenosides and included the rhizome, main root, branch root, and fibrous root.[5] Through quantitative comparison, it was found that the underground parts of *P. notoginseng* mainly contained PPD-type ginsenosides (Rb_1 and Rd) and PPT-type ginsenosides (Re, Rg_1, and R_1).[6] These major ginsenosides all exhibited great polarity due to the different types and amounts of sugar moieties attached to the C-3, C-6, and C-20 positions of the dammarane triterpenoid skeleton,[7] thus they were also known as polar ginsenosides. Through deglycosylation and dehydration etc., polar ginsenosides can be converted to less polar ginsenosides (minor ginsenosides).

Minor ginsenosides are considered functional molecules with various biological activities. For example, ginsenoside Rg_3 has prominent inhibitory effects on cancer cells (lung cancer cell line A549, melanoma cells, colon cancer cell line SW480, gallbladder cancer cells, etc.).[8] Ginsenosides Rh_1[9] and Rg_5[10] have important anti-inflammatory and antioxidant effects. Ginsenosides Rh_4[11] and F_4[12] play an anticancer role by inducing the apoptosis of cancer cells. However, the contents of minor ginsenosides in total ginsenosides of *P. notoginseng* are extremely low. The methods of transforming polar ginsenosides to minor ginsenosides have increasingly attracted attention. Well-known transformation methods include chemical transformation, steaming transformation, microwave degradation, and microbial transformation. Compared with other transformation methods, only the microbial transformation has the advantages of mild reaction conditions, substrate specificity, stable products, and being pollution free.[13]

In order to promote the application of minor ginsenosides in traditional medicine and functional food, a large number of microorganisms with the ability to transform ginsenosides have been discovered and applied. Some of the generally recognized as safe (GRAS) strains, such as *Lactobacillus paralimentarius* LH4,[14] *Lactobacillus rossiae* DC05,[15] *Aspergillus niger* XD101,[16] *Aspergillus tubingensis*,[17] and *Schizophyllum commune*,[18] can be directly used for the preparation of food grade minor ginsenosides. Nevertheless, the number of GRAS microorganisms with the ability to transform ginsenosides is fewer, and the types of transformation products are rare. To obtain microorganisms with stronger transformation ability and richer

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transformation products, it is inevitable for researchers to turn their attention to non-food-grade microorganisms. Further, recombining the genes with high ginsenoside-transforming activity in non-food-grade microorganisms into GRAS expression hosts will be beneficial to prepare various food-grade minor ginsenosides.[19] Therefore, non-food-grade microorganisms play a vital role in promoting the application of minor ginsenosides in traditional medicine and functional food.

In this study, *Talaromyces flavus* screened from *P. notoginseng* rhizosphere soil has the capacity to transform PPD-type and PPT-type ginsenosides in the underground parts of *P. notoginseng* to 18 minor ginsenosides. The transformation reactions invovled hydrolysis of outer and inner glucoses linked to C-20, outer glucose to C-3 in PPD-type ginsenosides, and inner glucose linked to C-20 in PPT-type ginsenosides; the generation of 20(*S*,*R*)-epimers by the reaction of epimerization; and the formation of C-20(21) and C-20(22) double-bond isomers by the reaction of dehydration. To the best of our knowledge, this transformation characteristic of *T. flavus* was first reported in fungi. Its crude enzyme can efficiently hydrolyze the outer glucoses linked to C-20 and C-3 in PPD-type ginsenosides within 48 h, suggesting that major ginsenosides Rb_2, Rb_3, and Rc in aerial (leaf and flower) parts of *P. notoginseng* may be transformed to minor ginsenosides by crude enzymes. The *T. flavus* and its crude enzyme are expected to be used for transforming major ginsenosides in the underground and aerial parts of *P. notoginseng* into minor ginsenosides, which greatly raises the functional and economic value of *P. notoginseng*.

MATERIALS AND METHODS

Materials. Authentic standards of ginsenosides Rg_1, Re, Rb_1, Rd, 20(*S*)-Rg_2, 20(*R*)-Rg_2, 20(*S*)-Rh_1, 20(*R*)-Rh_1, Rd, Rg_6, F_4, Rk_3, Rh_4, 20(*S*)-Rg_3, 20(*R*)-Rg_3, Rk_1, Rg_5, CK, 20(*S*)-Rh_2, 20(*R*)-Rh_2, and Rd_2 (C−O) and notoginsenosides R_1, 20(*S*)-R_2, 20(*R*)-R_2, Fa, Fd(C-Mx_1), Fe (C-Mc_1), and T_5 were purchased from the Sichuan Victory Biological Technology Co., Ltd. (Sichuan, China). The solvents methanol and acetonitrile for HPLC were purchased from Sigma-Aldrich Co. (St. Louis, MO, USA). The $HSGF_{254}$ silica gel TLC plate was purchased from Yantai Jiangyou Silicone Development Co., Ltd. (Shangdong, China). DEAE-cellulose DE52 was purchased from Shanghai Yuanye Bio-Technology Co., Ltd. (Shanghai, China). Other analytical-grade reagents were purchased from commercial sources.

Isolation and Cultivation of Fungus. Rhizosphere soil samples of triennial *P. notoginseng* were obtained from Kunming, Yunnan. Fungus was screened using selective Martins medium containing streptomycin for 5−7 days. If a pure colony appeared on the new PDA medium, it was numbered and stored at 4 °C for subsequent studies. The appearance of a pure colony may require multiple selections and cultures.

Screening of Fungus with the Ability to Transform Ginsenosides. The pure colony was cultured in 20 mL of PDB medium (pH 4.5−4.7), composed of 5 g/L potato extract powder and 15 g/L glucose. After culturing at 150 rpm/min and 25 °C for 5 days, the total ginsenosides (mixture of same mass of ginsenoside monomers Rb_1, Rd, Re, Rg_1, and notoginsenoside R_1) were added to the PDB medium. The concentration of total ginsenosides was 0.05 mg/mL. After substrate addition for incubation, the culture broth was harvested every 7 days. The ginsenosides in the culture broth were extracted by the same volume of aqueous saturated *n*-BuOH. The upper solution was used for TLC analysis, where in TLC analysis, $CHCl_3$−CH_3OH−H_2O (6.2:3.0:0.2, v/v/v) was used as a developing solvent, and 10% (v/v) H_2SO_4−ethanol was used as a chromogenic solvent. The upper solution was redissolved by chromatographic grade methanol after drying at 50 °C. To identify the types of minor ginsenosides in the transformation products, the samples were analyzed by HPLC, using a Shimazu LC-20AB high performance liquid chromatograph and a C_{18} HPLC column (5 μm, 250 × 4.6 mm, Welch, China). The analysis conditions were as follows: column temperature, 35 °C; flow rate, 1.0 mL/min; samples were detected by absorption at 203 nm; injection volume, 25 μL; the mobile phase was water (A) and acetonitrile (B) with gradient programs of 0−30 min (20% B), 30−60 min (20−36.5% B), 60−65 min (36.5−38% B), 65−70 min (38−45% B), 70−75 min (45−50% B), 75−90 min (50−56% B), 90−93 min (56−62% B), 93−103 min (62−75% B), 103−106 min (75−100% B).

Phylogenetic Analysis of Fungus with the Ability to Transform Ginsenosides. The amplification and sequencing of the ITS rDNA gene was completed by Kunming Branch of Tsingke Biotechnology Co., Ltd. ITS rDNA gene related taxonomic information was obtained from GenBank and National Center for Biotechnology Information (NCBI) servers. The phylogenetic tree was constructed by the neighbor-joining (NJ) methods in MEGA 6.0 software. The morphologies of conidiophores and ascospores were observed by optical microscopy and scanning electron microscopy, respectively.

Biotransformation Pathways. Notoginsenoside monomer R_1 and ginsenoside monomers Rg_1, Re, Rb_1, and Rd were incubated with active fungus at 25 °C and 150 rpm/min for 18−21 days. Each transformation experiment of ginsenoside monomer consisted of three groups (substrate control group, containing substrate and medium; microorganism control group, containing fungus and medium; ginsenoside transformation group, containing fungus, substrate, and medium). The concentration of ginsenoside monomer was 0.05 mg/mL. TLC, HPLC, and MS were used to identify the types of minor ginsenosides in the transformation products. On the basis of the above analysis, the biotransformation pathways of major ginsenosides in the underground parts of *P. notoginseng* were proposed. MS analysis was performed with Agilent quadrupole-time-of-flight MS (Q-TOF-MS) equipped with an electrospray ionization source under negative ion mode. The following conditions were used: a drying gas N_2 flow rate of 8 L/min, a cone gas temperature of 350 °C, and a nebulizer pressure of 35 psig. TLC and HPLC analysis conditions were performed as described in Method 2.3.

Dynamic Change of Minor Ginsenosides Contents in the Transformation Products of Notoginsenoside R_1 and Ginsenosides Rb_1 and Rg_1. Monitoring for minor ginsenosides in the transformation products of characteristic R_1, PPD-type ginsenoside Rb_1, and PPT-type ginsenoside Rg_1 via HPLC could help clarify the duration reaching maximal concentration. Through the dilution method of the mother liquor, authentic minor ginsenosides were diluted to prepare seven standard solutions with different concentrations. The calibration curves were established according to the relationship between peak area and ginsenoside concentrations. To investigate the precision of the instrument, the same authentic ginsenosides solution was analyzed six times, and the RSD value of the peak area was calculated. To investigate the stability of minor ginsenosides in sample solution, the same sample was analyzed every 4 h six times, and the RSD value of the peak area was calculated. To

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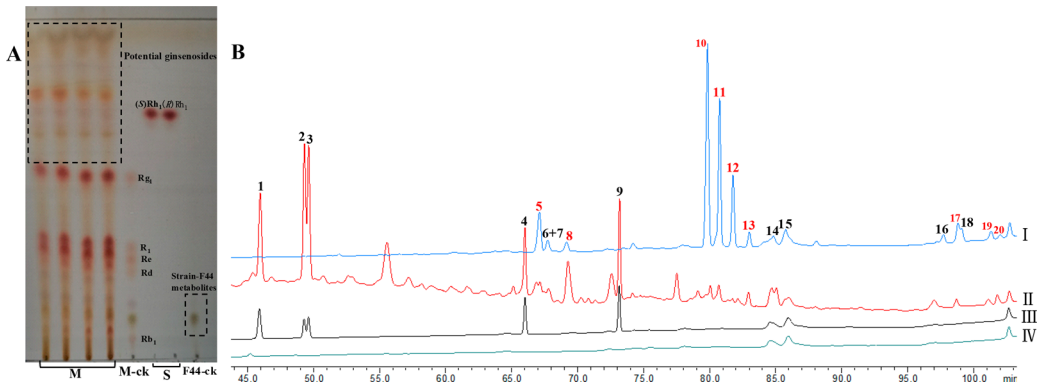

Figure 1. TLC and HPLC analysis of transformation products of mixture of five saponins by strain-F44. (A) M, the transformation products; M-ck, the control of saponin mixture; S, authentic ginsenosides; F44-ck, the control of strain-F44. Developing solvent is $CHCl_3$−CH_3OH−H_2O (6.2:3.0:0.2, v/v/v). (B; I) Mixture of 15 authentic minor ginsenosides; (II) transformation products; (III) the control of saponin mixture; (IV) the control of strain-F44. The peaks: 1, R_1; 2, Rg_1; 3, Re; 4, Rb_1; 5, 20(*S*)-Rg_2; 6 + 7, 20(*S*)-Rh_1 + 20(*R*)-Rg_2; 8, 20(*R*)-Rh_1; 9, Rd; 10, Rg_6; 11, F_4; 12, Rk_3; 13, Rh_4; 14, 20(*S*)-Rg_3; 15, 20(*R*)-Rg_3; 16, Rk_1;17, Rg_5; 18, C−K; 19, 20(*S*)-Rh_2; 20, 20(*R*)-Rh_2.

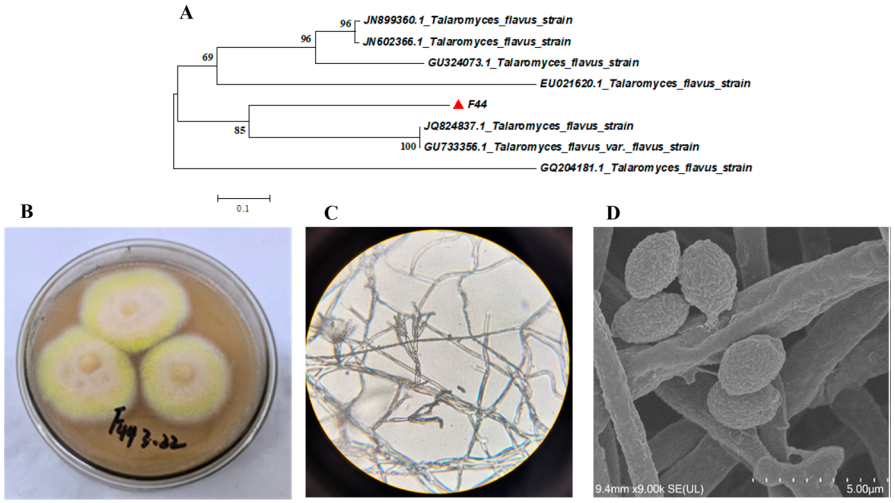

Figure 2. ITS gene and morphology identification of strain-F44. (A) The neighbor-joining tree based on ITS rDNA gene sequences of strain-F44. (B) Morphology of strain-F44. (C) Conidiophore of strain-F44. (D) Ascospores of strain-F44; scale bars, 5 μm.

investigate the repeatability of the results, six duplicate samples were analyzed and the RSD value of the peak area was calculated. The recovery test was performed by spiking a sample with mixed standards. The recovery rate formula is as follows and the effective range should be between 95−105%:

$$\text{recovery rate (95 - 105\%)} = (\text{observed amount} - \text{original amount})/\text{spiked amount} \times 100\%$$

Notoginsenoside monomer R_1 and ginsenoside monomers Rb_1 and Rg_1 were respectively incubated with fungus. Each ginsenoside transformation group was performed in triplicate. The same volume of culture broth was removed at 3 day intervals for quantitative analysis via HPLC. The quantitative results were presented as the mean ± standard deviation.

Preparation and Activity Analysis for Extracellular Crude Enzymes. Total ginsenosides (same as described in Method 2.3) were used to induce the secretion of fungal extracellular enzymes. The active fungus was cultured in 800 mL of PDB medium containing 5 mg of total ginsenosides. The air filtered through a 0.22 μm membrane was continuously pumped into the suspension. After 14−21 days of culture, twice the volume of 20 mM acetate buffer (pH 5.0) was added to a culture broth, and the mixed solution was gently stirred for 2 h. The culture broth was filtered by four layers of gauze. The filtrate was centrifuged at 10 000 *g* for 10 min to remove cells. Solid ammonium sulfate was added to the supernatant. In order to precipitate the protein, the saturation of ammonium sulfate was upregulated to 85%. The crude enzyme was prepared by centrifuging at 10 000 *g* for 10 min, resuspension in 20 mM acetate buffer (pH 5.0), recentrifuging to remove insoluble protein at 10 000 *g* for 10 min, dialyzing at a molecular mass cutoff of 14 kDa, and freeze-drying. Two milligrams of crude enzyme was incubated in 20 mM acetate buffer (pH 5.0) at 30 °C for 48 h with 0.25 μM R_1, Rg_1, Rb_1, Rb_2, Rb_3, Rc, 20(*S*, *R*)-Rg_3, and Fa. The enzyme transformation products were analyzed via TLC and HPLC. One unit of glucosidase activity was defined as the quantity of enzyme required to hydrolyze 1 nM of ginsenoside Rb_1 per hour at pH 4.5 and 50 °C.

Temperature and pH are two main factors to influence the enzymatic activity. To explore the effects of different pH and temperatures on enzymatic activity, 2 mg of crude enzyme containing 0.3 U of glucosidase activity and 0.25 μM ginsenoside Rb_1 were incubated at pH and temperatures ranges of 3.5−8.5 and 25−70 °C for 48 h. Each experiment was triplicated.

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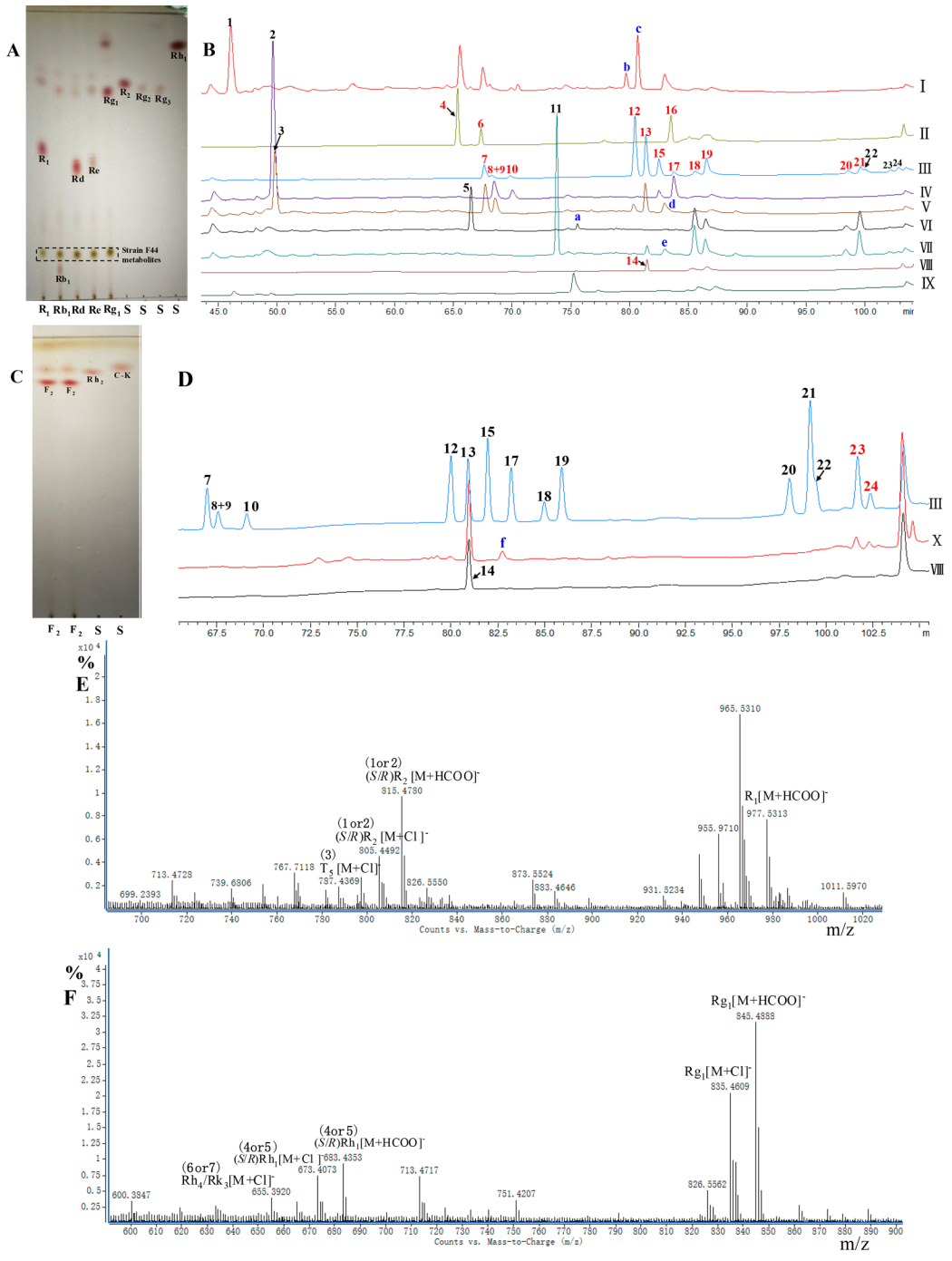

Figure 3. continued

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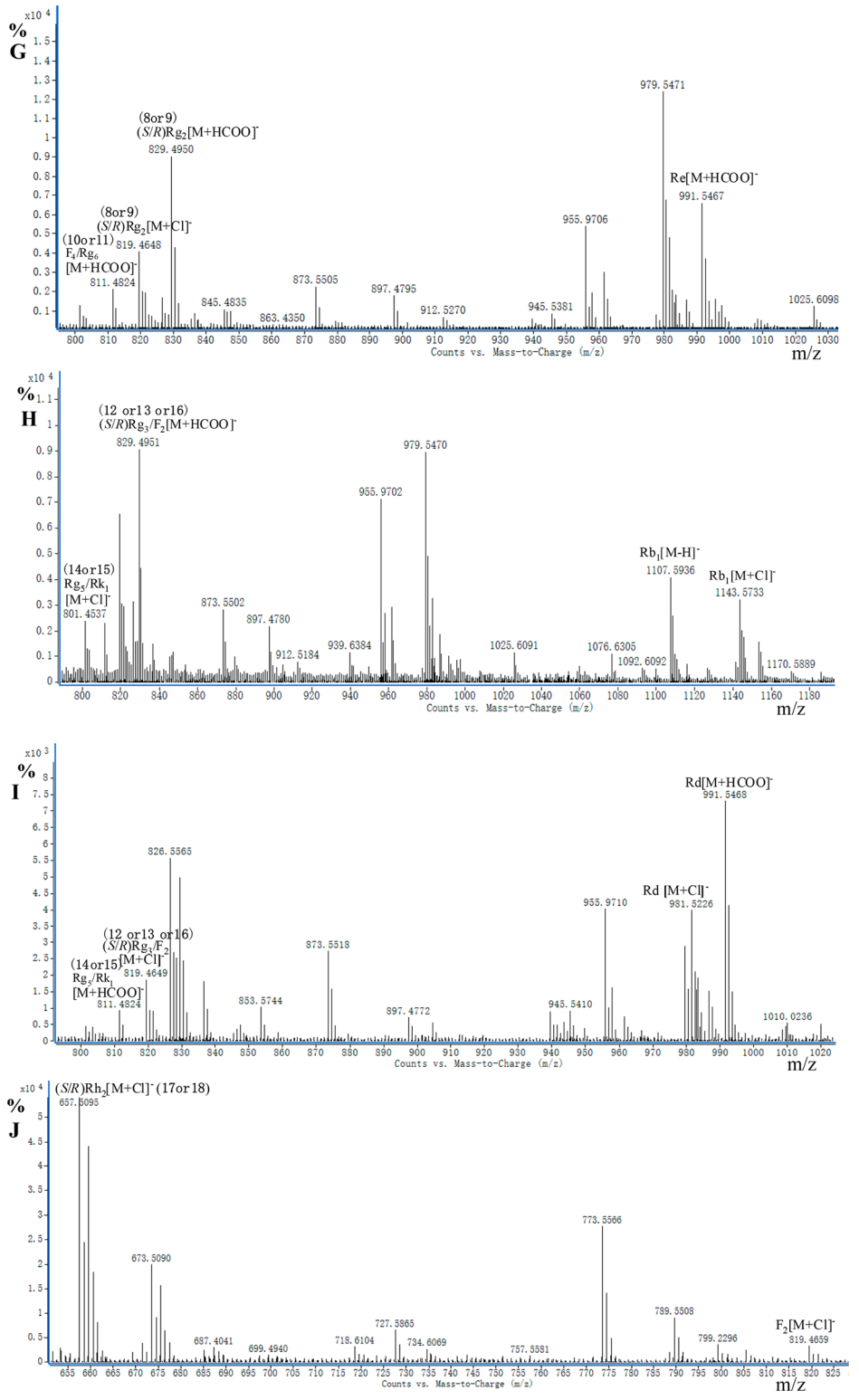

Figure 3. TLC, HPLC, and MS analysis of transformation products of six monomer saponins by *T. flavus*. (A and C) TLC analysis of transformation products of monomer saponins R_1, Rb_1, Rd, Re, Rg_1, and F_2. S, authentic ginsenosides. Developing solvent is $CHCl_3$−CH_3OH−H_2O (6.2:3.0:0.2, v/v/v). (B and D) HPLC analysis of transformation products of R_1 [I]; three authentic notoginsenosides [II]; 15 authentic ginsenosides [III]; transformation products of ginsenoside Rg_1 [IV], Re [V], Rb_1 [VI], and Rd [VII]; authentic ginsenoside F_2 [VIII]; and the control of *T. flavus* [IX]; transformation products of ginsenoside F_2 [X]. The peaks: 1, R_1; 2, Rg_1; 3, Re; 4, 20(*S*)-R_2; 5, Rb_1; 6, 20(*R*)-R_2; 7, 20(*S*)-Rg_2; 8 +9, 20(*S*)-Rh_1 + 20(*R*)-Rg_2; 10, 20(*R*)-Rh_1; 11, Rd; 12, Rg_6; 13, F_4; 14, F_2; 15,Rk_3; 16, T_5; 17, Rh_4; 18, 20(*S*)-Rg_3; 19, 20(*R*)-Rg_3; 20, Rk_1; 21, Rg_5; 22, C−K; 23, 20(*S*)-Rh_2; 24, 20(*R*)-Rh_2. a, b, c, d, e, and f: Potential ginsenoside derivatives. ESI-MS spectra (negative ion mode) of the transformation products of notoginsenoside R_1 (E), ginsenosides Rg_1 (F), Re (G), Rb_1 (H), Rd (I), and F_2 (J) by *T. flavus*.

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Ginsenoside Rb_1 consumption was quantified by HPLC and presented as mean ± standard deviation.

Molecular Mass Measurement of Purified Glucosidase. Twenty milligrams of crude enzyme was dissolved in 2 mL of 20 mM acetate buffer (pH 5.0). The enzyme solution was gradually eluted using a DEAE-Cellulose DE-52 column (ϕ1.5 cm × 15 cm). The proteins were fractionated stepwise with 0.11, 0.14, 0.17, 0.21, 0.27, and 0.34 M KCl−acetate buffer (20 mM, pH 5.0). Each 3 mL eluent was taken as a fraction. The proteins in fractions were detected by absorption at 280 nm via UV−visible spectrophotometer (Shimazu UV-2600). The hydrolysis activity of the proteins was analyzed by incubation of ginsenosides Rb_1 and Rg_1 with proteins. The incubation products were analyzed via TLC. The molecular mass of active proteins was measured by SDS-PAGE using 5% and 10% (w/v) stacking polyacrylamide and separating gels. The linear regression equation was solved using standard proteins: aprotinin (6.5 kDa), lysozyme (14.3 kDa), trypsin inhibitor (20.1 kDa), carbonic anhydrase (29.0 kDa), ovalbumin (44.3 kDa), serum albumin (66.4 kDa), phosphorylase b (97.2 kDa), β-galactosidase (116 kDa), and myosin (200 kDa). Protein bands were visualized via Coomassie brilliant blue R-250. The linear regression relationship between molecular mass and mobility is as follows:

$$\lg(\mathrm{MW}) = K - bm_r$$

where MW is the molecular mass of the protein, m_r is the relative mobility (moving distance of protein/moving distance of bromophenol blue indicator), K is the intercept, and b is the slope.

RESULTS

Screening of Fungus with the Ability to Transform Ginsenosides. TLC analysis of transformation products by fungi showed that strain-F44 has an outstanding ability to transform total ginsenosides (mixture of same mass of monomer ginsenosides Rb_1, Rd, Re, and Rg_1 and notoginsenoside R_1). Figure 1A showed that there were many points with a large Rf value above the substrate. It indicated that the substrate may be transformed. Furthermore, HPLC analysis of transformation products by strain-F44 showed that the transformation products may include minor ginsenosides 20(*S*)-Rg_2, 20(*R*)-Rh_1, Rg_6, F_4, Rk_3, Rh_4, Rg_5, 20(*S*)-Rh_2, 20(*R*)-Rh_2, etc., seen in Figure 1B.

Table 1. Summary of Transformation Products of Saponin Monomer by *T. flavus*

substrates	identified products
Rb_1	**12**, **13**, **14**, **15**
Rd	**16**, **12**, **13**, **14**, **15**
F_2	**17**, **18**
Re	**8**, **9**, **10**, **11**
Rg_1	**4**, **5**, **6**, **7**
R_1	**1**, **2**, **3**

Phylogenetic Analysis of Strain-F44 with the Ability to Transform Ginsenosides. On the basis of the sequencing of the ITS rDNA gene and a comparison in the GenBank database, it was found that strain-F44 belonged to the genus *Talaromyces* and exhibited significant similarity to *Talaromyces flavus* in Figure 2A. After strain-F44 was cultured on a PDA medium for 4 days, the following colony characteristics were observed: the colony surface was white and light yellow; the texture, floccose; and the edge, neat, as in Figure 2B. Morphological characteristics were as follows: conidiophores exhibited biverticillate penicilli, as in Figure 2C. The ascospores were ellipsoidal in size (2−3 × 1−2 μm) and have surficial protuberances, as in Figure 2D. The above results were consistent with the description of the *Talaromyces flavus* (CBS 310.38^T) by Yilmaz et al.[20] *Talaromyces flavus*-F44 was stored in the China General Microbiological Culture Collection Center (Beijing, China), and the collection number was CGMCC NO.22438.

Biotransformation Pathways of Major Ginsenosides in the Underground Parts of *P. notoginseng* by *T. flavus*. TLC analysis of the transformation products by *T. flavus* showed that there were new points with a large Rf value above each substrate in Figure 3A. These results indicated that the *T. flavus* can transform ginsenosides Rb_1, Rd, Re, and Rg_1 and notoginsenoside R_1 to minor ginsenosides. A comprehensive HPLC analysis of the transformation products by *T. flavus* showed that each polar ginsenoside was transformed to several minor ginsenosides in Figure 3B. Compared to multiple authentic standards, compounds **1** to **18** were assignable to **1**, 20(*S*)-R_2; **2**, 20(*R*)-R_2; **3**, T_5; **4**, 20(*S*)-Rh_1; **5**, 20(*R*)-Rh_1; **6**, Rh_4; **7**, Rk_3; **8**, 20(*R*)-Rg_2; **9**, 20(*R*)-Rg_2; **10**, F_4; **11**, Rg_6; **12**, 20(*S*)-Rg_3; **13**, 20(*R*)-Rg_3; **14**, Rg_5; **15**, Rk_1; **16**, F_2; **17**, 20(*S*)-Rh_2; and **18**, 20(*R*)-Rh_2. Interestingly, ginsenoside F_2 (**16**) was found in the transformation products of ginsenoside Rd but not in those of ginsenoside Rb_1. Beyond that, its deglycosylated products (ginsenoside Rh_2 or CK) were not found in the transformation products of ginsenoside Rd. It may be related to the inhibitory effects of intermediates.[21] In order to ascertain whether new deglycosylated products are produced, ginsenoside monomer **16** was directly incubated with *T. flavus*. TLC (Figure 3C) and HPLC (Figure 3D) analysis of transformation products of ginsenoside **16** by *T. flavus* showed that ginsenoside **16** can be transformed to compounds **17** and **18** but not to ginsenoside C−K. The above results indicated that the transformation pathway Rb_1 → Rd→ **16** → compounds **17** and **18** existed in *T. flavus*. Maybe the inhibition of intermediates made it so that the pathway was weakly present in *T. flavus*. Last, through scanning the transformation products of six monomer ginsenosides via Q-TOF-MS (Figure 3E−J), it was found that the mass-to-charge ratio of substrates and products can be matched. The summary of transformation products of saponin monomers was shown in Table 1. According to refs 22 and 23, epimerization may occur immediately after the hydrolysis of inner glucose on C-20. It should be noted that the tertiary hydroxyl groups with different configurations on C-20 may occur through nonselective attack of the OH group on the trigonal planar sp^2 hybridized carbenium intermediate.[24] Subsequently, C-20(21) and C-20(22) double-bond isomers were generated through dehydration of the tertiary hydroxy group. Therefore, the proposed biotransformation pathways of major ginsenosides in the underground parts of *P. notoginseng* by *T. flavus* were as follows: Rb_1 → Rd→ compounds **12** and **13** → compounds **14** and **15** and Rb_1 → Rd → compound **16** → compounds **17** and **18** (Figure 4A); Re → compounds **8** and **9** → compounds **10** and **11** (Figure 4B); Rg_1 → compounds **4** and **5** → compounds **6** and **7** (Figure 4C); R_1 → compounds **1** and **2** → compound **3** (Figure 4D).

Dynamic Change of Minor Ginsenosides Contents in the Transformation Products of Notoginsenoside R_1 and Ginsenosides Rb_1 and Rg_1 by *T. flavus*. The validation data of the quantitative method for ginsenosides are in Table S1. The alteration trend of minor ginsenosides in transformation

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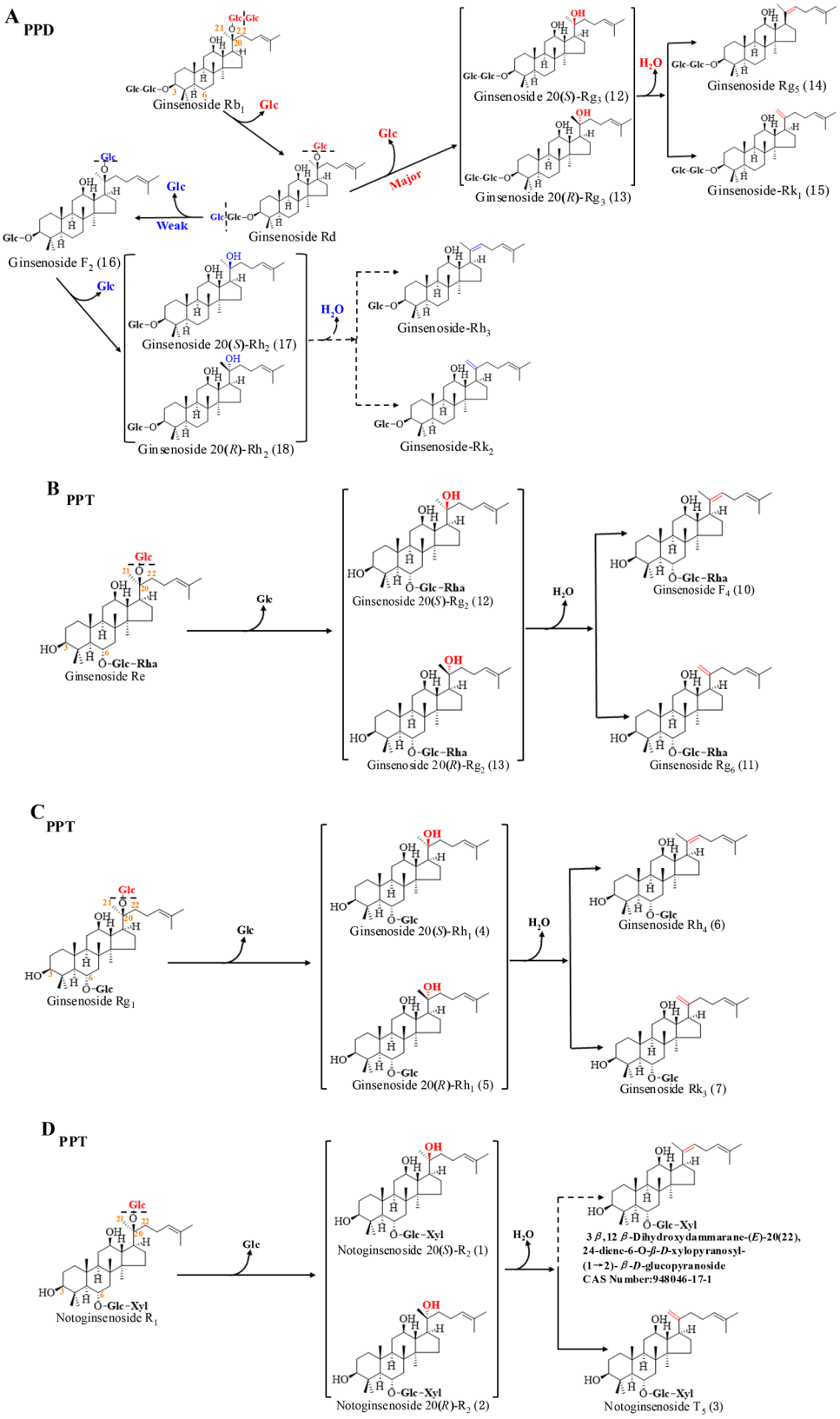

Figure 4. Proposed biotransformation pathways of ginsenosides Rb_1 and Rd (A), Re (B), and Rg_1 (C) and notoginsenoside R_1 (D) by *T. flavus*. Solid lines represent the transformation pathways proposed in this study. Dotted lines represent the transformation pathways predicted by theory.

products of notoginsenoside R_1 and ginsenosides Rb_1 and Rg_1 by *T. flavus* is shown in Figure 5. According to Figure 5A, the concentration of compound **1** was more than that of compound **2** in the transformation products. The maximal concentration of compound **1** occurred on the 12th day. Similarly, according to Figure 5B, the concentration of compound **12** was more than that of compound **13**. The maximal concentration of compound **12** occurred on the 12th day. In Figure 5C, the concentration of

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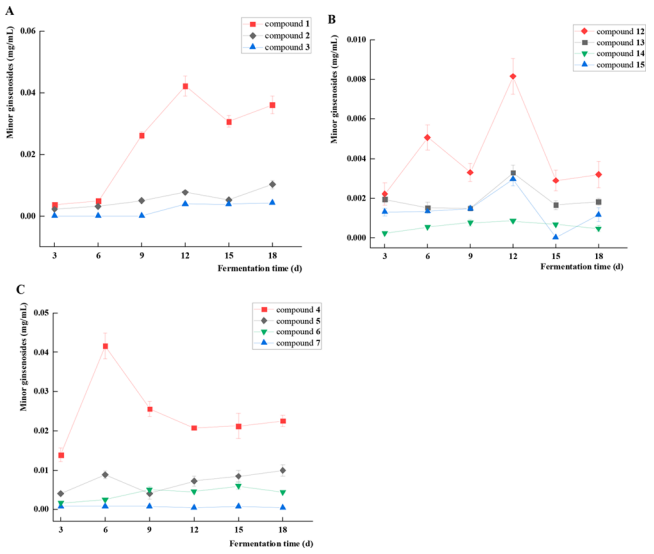

Figure 5. Dynamic change of minor ginsenoside contents in the transformation product of monomer notoginsenoside R_1 (A), ginsenosides Rb_1 (B), and Rg_1 (C) by *T. flavus*.

compound **4** was more than that of compound **5**. The maximal concentration of compound **4** occurred on the sixth day.

Activity Analysis of Extracellular Crude Enzymes of *T. flavus*. Notoginsenoside monomer R_1 and ginsenoside monomers Rg_1 and Rb_1 were used as substrates to incubate the extracellular crude enzymes of *T. flavus*. According to Figure 6A, the extracellular crude enzymes of *T. flavus* only hydrolyzed the outer glucoses linked to C-20 and C-3 of ginsenoside Rb_1 but not glucoses of notoginsenoside R_1 and ginsenoside Rg_1. In order to verify this conclusion, ginsenoside monomers Rb_2, Rb_3, Rc, 20(*S*,*R*)-Rg_3, and notoginsenoside Fa (they all have outer glucose linked to the C-3 position) were incubated with the crude enzymes. According to Figure 6B, it was found that there were new points above each saponin monomer except 20(*R*)-Rg_3. As Figure 6C, HPLC analysis of the transformation products showed that the outer glucoses linked to C-20 and C-3 of ginsenosides Rb_1, Rb_2, Rb_3, Rc, and 20(*S*)-Rg_3 (except 20(*R*)-Rg_3) can be hydrolyzed by extracellular crude enzymes of *T. flavus*. Compared to multiple authentic standards, compounds **19** to **22** were assignable to **19**, Gyp-XVII; **20**, C−O; **21**, C-Mx_1; **22**, C-Mc_1. The proposed transformation pathways by crude enzymes are as follows: Rb_1 → Rd/compound **19** → compound **16**; Rb_2 → compound **20**; Rb_3 → compound **21**; Rc → compound **22**; 20(*S*)-Rg_3 → compound **17**. According to Figure 6D−G, the crude enzyme activity was better at pH 4.5 and 50 °C. The conversion rate of ginsenoside Rb_1 reached the maximum limit (~80%)

Molecular Mass of Purified Glucosidase. Figure S1 showed that the crude enzyme contained multiple protein bands, but the purified enzyme with hydrolytic activity was a single band. Moreover, the band of purified enzyme can be found at the same position of the crude enzyme. Through calculating the protein mobility, the molecular mass of the glucosidase was about 64 kDa.

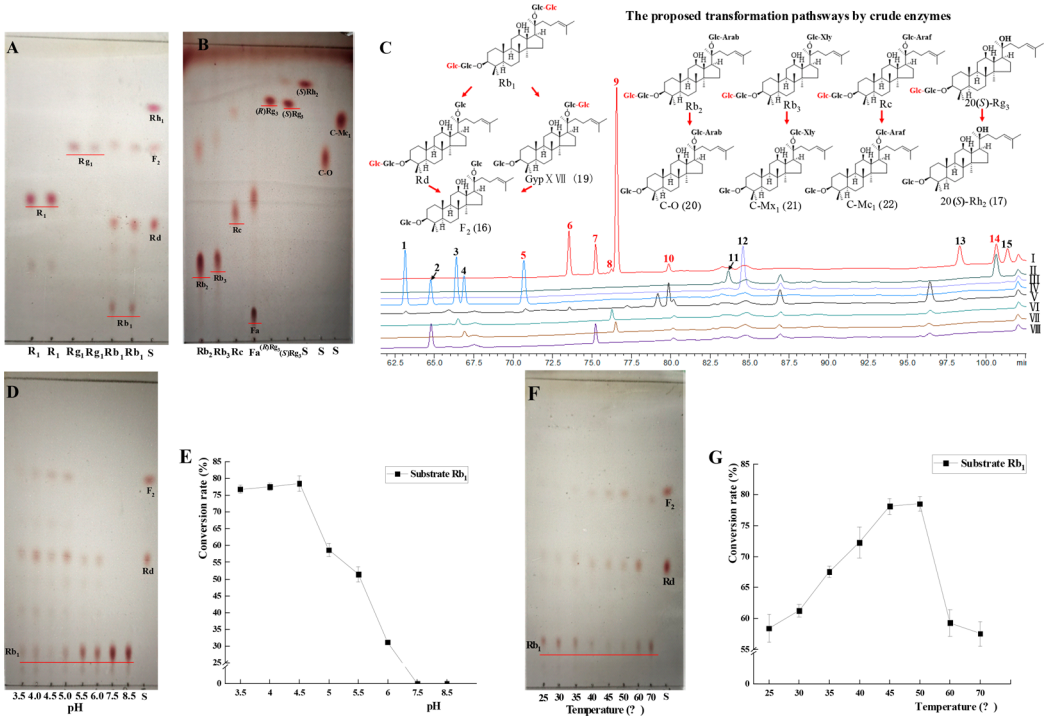

Figure 6. Activity analysis of extracellular crude enzymes of *T. flavus*. (A, B) TLC analysis of transformation products of R_1, Rg_1, Rb_1, Rb_2, Rb_3, Rc, Fa, 20(*R*)-Rg_3, and 20(*S*)-Rg_3 by extracellular enzymes. (C) HPLC analysis of eight authentic minor ginsenosides [I]; transformation products of 20(*R*)-Rg_3 [II] and 20(*S*)-Rg_3 [III]; five authentic polar ginsenosides [IV]; and transformation products of Rb_1 [V], Rb_2 [VI], Rb_3 [VII], and Rc[VIII]. The peaks: 1, Rb_1; 2, Rc; 3, Rb_2; 4, Rb_3; 5, Rd; 6, GypXVII; 7, C-Mc_1; 8, C−O; 9, C-Mx_1; 10, F_2; 11, 20(*S*)-Rg_3; 12, 20(*R*)-Rg_3; 13, C−K; 14, 20(*S*)-Rh_2; 15, 20(*R*)-Rh_2. Influence of different pH's (D, E) and temperatures (F, G) on the hydrolysis of ginsenoside Rb_1 by crude enzymes. In all TLC analyses, S was authentic ginsenosides, and developing solvent was $CHCl_3$−CH_3OH−H_2O (6.2:3.0:0.2, v/v/v).

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DISSCUSSION

In this study, *T. flavus* was screened from the *P. notoginseng* rhizosphere, which could transform major ginsenosides in the underground parts of *P. notoginseng* to minor ginsenosides. Through comprehensive analysis, it was found that *T. flavus* exhibited the following three activities: hydrolysis of outer and inner glucoses linked to C-20, outer glucose to C-3 in PPD-type ginsenosides and inner glucose linked to C-20 in PPT-type ginsenosides; generation of 20(*S*,*R*)-epimers by epimerization; and formation of C-20(21) and C-20(22) double-bond isomers by dehydration. By referring to previous studies, we found that similar transformation activities may exist in the *Lactobacillus plantarum*[25] but not in fungi. However, the transformation substrate was the crude extract of *Panax ginseng* with various ginsenosides, resulting in a lack of sufficient evidence to support the proposed ginsenoside transformation pathway. Thus, the transformation activities of *T. flavus* found in this study were first reported in fungi, and its transformation pathways are supported by enough evidence.

According to the HPLC analysis of transformation products of ginsenoside monomers by *T. flavus*, the chromatographic peaks a, b, c, d, e, and f may be potential ginsenoside derivatives because the retention times of these peaks were near those of minor ginsenosides. If the ginsenoside monomers are fermented by *T. flavus* on a large scale and products are isolated and purified, new ginsenoside derivatives could be found via spectroscopic methods.

On the basis of the results that ginsenosides Rb_1, Rd, Re, and Rg_1 can be transformed to 20(*S*,*R*)-epimers, C-20(21) and C-20(22) double-bond isomers by *T. flavus*, we inferred that notoginsenoside 20(*S*,*R*)-R_2 could be transformed not only to the notoginsenoside T_5 with a C-20(21) double bond but also to 3β,12β-dihydroxydammarane-(*E*)-20(22),24-diene-6-*O*-β-D-xylopyranosyl-(1→2)-β-D-glucopyranoside with a C-20(22) double bond.[26] Moreover, we also inferred that ginsenoside 20(*S*, *R*)-Rh_2 from ginsenoside F_2 could be transformed to ginsenoside Rk_2 with a C-20(21) double bond and ginsenoside Rh_3 with a C-20(22) double bond by *T. flavus*.

Compared with various transformation products and pathways of *T. flavus*, its crude enzymes exhibited high regiospecificity for hydrolysis of ginsenosides; i.e., it could only hydrolyze the outer glucoses linked to C-20 and C-3 in PPD-type ginsenosides. It could be the inactivation of certain subunits of the glucosidase, during the preparation of crude enzymes.

The transformation of polar ginsenosides to minor ginsenosides by microoganisms is closely related to the detoxification of pathogens. In order to successfully invade the host plant, pathogens can produce some glycosylhydrolases to degrade the resistance chemicals (ginsenoside, α-tomatine, α-solanine, α-chaconine, etc.) of the plant.[27] It was reported that after *Ilyonectria mors-panacis* G3B, a pathogen of *P. notoginseng* root rot, was treated with ginsenoside Rb_1 and Rg_1 for 4 and 12 h, genes encoding saponin-detoxifying enzymes (glycosylhydrolase, oxidoredutase, transporter, etc.) were simultaneously highly expressed in pathogen G3B via high-throughput RNA-seq.[28] By using a similar ginsenoside treatment and high-throughput RNA-seq to *T. flavus*, genes encoding enzymes that catalyze the C-20 position epimerization of ginsenoside and C-20 position dehydration of ginsenoside may be mined. Further, cloning, expression, and verification of these functional genes will promote their application in the targeted modification of ginsenoside structure.

ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acsomega.2c00557.

(Figure S1) Glucosidase in SDS-PAGE. Marker, marker protein: aprotinin (6.5 kDa), lysozyme (14.3 kDa), trypsin inhibitor (20.1 kDa), carbonic anhydrase (29.0 kDa), ovalbumin (44.3 kDa), serum albumin (66.4 kDa), phosphorylase b (97.2 kDa), β-galactosidase (116 kDa), and myosin (200 kDa). Crude, crude enzyme. Pure, purified glucosidase. (Table S1) Validation data of quantitative method for 12 saponins (PDF)

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Notes

The authors declare no competing financial interest.

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ABBREVIATIONS

PPD, protopanaxadiol; PPT, protopanaxatriol; CK, ginsenoside compound K; TLC, thin layer chromatography; HPLC, high performance liquid chromatography; MS, mass spectrometry; PDA, potato dextrose agar; PDB, potato dextrose broth; ITS,

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internal transcribed spacer; RSD, relative standard deviation; SDS-PAGE, sodium dodecyl sulfate−polyacrylamide gel electrophoresis

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