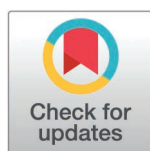# PLOS ONE

RESEARCH ARTICLE

The transformation pathways and optimization of conditions for preparation minor ginsenosides from *Panax notoginseng* root by the fungus *Aspergillus tubingensis*

Fei-Xing Li[1ᵒ], **Dong-Mei Lin**[1ᵒ], **Jin Yang**[1], **Xiu-Ming Cui**[1], **Xiao-Yan Yang**[1,2]*

1 Faculty of Life Science and Technology, Kunming University of Science and Technology, Kunming, Yunnan, China, **2** Yunnan Key Laboratory of Sustainable Utilization of Panax Notoginseng, Kunming, Yunnan, China

ᵒ These authors contributed equally to this work.
* yangxy@kust.edu.cn

Abstract

Minor ginsenosides exhibit enhanced pharmacological effects in comparison to the major ginsenosides. However, the natural content of minor ginsenosides in plants is typically insufficient to satisfy clinical demand. Therefore, we investigated the biotransformation of the major ginsenosides in *Panax notoginseng* to minor ginsenosides by the fungus *Aspergillus tubingensis*. The transformation products were analyzed using TLC, HPLC, and LC-MS techniques to propose the biotransformation pathways of major ginsenosides. *A. tubingensis* was found to transform the main ginsenosides into 15 minor ginsenosides, inculding (R/S)-Rg_3, Rk_1, Rg_5, F_2, (R/S)-Rh_1, Rk_3, Rh_4, (R/S)-Rg_2, F_4, Rg_6 and (R/S)-R_2. The transformation reactions encompassed isomerization, hydrolysis and dehydration. We have also optimized the reaction temperature and pH for the crude enzyme extracted from this fungus, which has a molecular weight of $66\,kDa$. Based on our current knowledge, this transformative characteristic of *A. tubingensis* was initially documented for the concurrent transformation of PPD and PPT type saponins in *P. notoginseng*. This method of preparing minor saponins will be valuable for the development of *P. notoginseng* as a traditional medicinal material.

1. Introduction

Panax notoginseng (Burk.) F. H. Chen is a highly famous traditional Chinese medicinal materials. Its medicinal parts are the dried roots and rhizomes, and the main bioactive ingredients of *P. notoginseng* are saponins, which exhibit a variety of pharmacological activities [1–2]. The primary ingredient of *P. notoginseng* root are protopanaxadiol (PPD)-type ginsenosides and protopanaxatriol (PPT)-type ginsenosides [3–4]. Among these saponins, the ginsenosides Rb_1, Rd, Re and Rg_1, as well as the notoginsenoside R_1, account for over 80% of the total saponins [5], and they are therefore known as major ginsenosides [6]. These major ginsenosides tend to be greater polarity due to the presence of more sugar groups at positions C-3, C-6 and C-20 of the ginsensides. Because of this polarity, orally administered saponins are not readily absorbed

in the small intestine and thus are characterized by low bioavailability. Therefore, to enhance the bioavailability of these saponins, it is necessary to remove glucopyranosyl at C-3, C-6 and C-20 through hydrolysis to generate minor ginsenosides with fewer glycosyl groups. As expected, the pharmacological activities of these minor ginsenosides, including Rg_3, Rh_1, and Rh_2, have been shown to be higher than those of the main naturally occurring ginsenosides [7–9]. In particular, the minor ginsenosides Rg_3, Rg_2, Rh_4, C-K and F_2 have been found to exert anti-cancer activity [10–14], and minor ginsenosides Rh_2, Rk_1, Rk_3, Rg_5 and Rg_6 exert anti-tumor activity [15–19].

Obtaining significant amounts of minor ginsenosides is challenging by isolation from *P. notoginseng* root because of their low abundance. Due to the fact that minor ginsenosides and major ginsenosides share the same parent nucleus in their structures, so they can be derived from major saponins. One key way to obtain these minor ginsenosides involves biological transformation, which relies on microbial strains or enzymes produced by them to hydrolyze, dehydrate, oxidize and isomerize the substrate saponins, so as to obtain minor saponins with high pharmacological activity. Numerous studies have investigated the biological transformation of major saponins into minor ginsenosides. As an illustration, ginsenoside Rb_1 can be transformed to Rd, F_2 and C-K by *Aspergillus niger* [20]. In addition, the PPD-type saponins were found to be transformed into C-K with higher pharmacological activity, by *A. tubingensis* isolated from fermented soybean [21]. Ginsenoside Rb_2 can be transformed to C-O, C-Y and C-K by ginsenosidase type-I from *A. niger* g.848 [22], and ginsenosides Rb_1, Rb_2, Rc, Rd and Rg_3 were found to be completely transformed into Gyp-XVII, C-O, $C-Mc_1$, F_2 and Rh_2, respectively, by *A. oryzae* [23]. Overall, due to its efficient product generation, mild reaction conditions and high conversion rate, biological transformation represents an important tool in ginsenoside transformation [24,25].

In this study, the properties of the biological transformations of ginsenosides Rb_1, Rd, Re and Rg_1 and notoginsenoside R_1 by *A. tubingensis* were studied. The transformation products of major ginsenosides were analyzed using chromatographic and mass spectrometric methods, leading to the proposal of biotransformation pathways for major ginsenosides. Furthermore, the enzyme responsible for these transformations, β-ginsenosidase, was purified and characterized. We found that the enzyme isolated from *A. tubingensis* was over a wide temperature range and the enzyme activity remained above 70% which formed the basis for its large-scale preparation.

2. Materials and methods

2.1. Materials

The standard ginsenosides Rb_1, Rd, R_1, Rg_1, Re, (*R/S*)-R_2, (*R/S*)-Rg_2, (*R/S*)-Rh_1, (*R/S*)-Rg_3, T_5, Rg_6, F_4, Rk_3, Rh_4, F_2, CK, Rk_1 and Rg_5 were purchased from Vicky Biotechnology Co., Ltd., China. DEAE-52 was purchased from Shanghai Yuan ye Biological Co., Ltd., China. The BCA protein concentration assay kit was purchased from Beyotime Biotechnology, China. *A. tubingensis* was isolated from soil in which *P. notoginseng* was growing. The standard proteins were purchased from Takara Bio, Inc. (Otsu, Shiga, Japan). Column chromatography (CC) was performed using a macroporous resin D101 (Shanghai Yuanye Bio-Technology Co., Ltd, China), silica gel (200-300 mesh; Qingdao Marine Chemical Ltd., China), RP-18 gel (40-75 μm, Fuji Silysia Chemical Ltd., Japan), and Sephadex LH-20 (Amersham Biosciences, Sweden). Fractions were monitored by TLC (GF 254, Qingdao Haiyang Chemical Co., Ltd. Qingdao).

P. notoginseng was collected from Wenshan County, Yunnan Province, China, in August 2022, and identified by Prof Xiuming Cui, Kunming University of Science and Technology. The voucher specimen is No. YXY20220820. The sample collection site is our school's

experimental research base. All of our research has been approved by the Kunming University of Science and Technology.

2.2. Isolation and identification of fungus

A. tubingensis was isolated from the cultivation soil of *P. notoginseng* in the Laboratory Greenhouse for *P. notoginseng* cultivation, Kunming University of Science and Technology, China. The RSA login number of the strain was recorded with NCBI as SRR2285937. Morphological characteristics of *A. tubingensis* and its spores were observed visually and recorded, and the spore morphology was observed by microscopy. The Kunming Branch of Tsingke Biotechnology Co., Ltd. conducted the amplification and sequencing of the internal transcribed spacer (ITS) rDNA gene. The sequencing results were uploaded to NCBI for comparison. Utilizing MEGA 7.0, a Neighbor-Joining (NJ) phylogenetic tree was constructed by selecting species exhibiting significant homology.

2.3. Isolation and purification of five monomeric saponins from total saponins of *P. notoginseng* root

After crushing the main root of *P. notoginseng* (200 g), the main saponins were extracted by ethanol reflux extraction [26]. This extraction process utilized a 75% ethanol solution and a solid-liquid ratio of 1:10. After extraction, solid-liquid separation was carried out, and the separated liquid was extracted by adding 2 times volume of water-saturated n-butanol. The upper liquid was used for vacuum evaporation and concentration to obtain the crude extract of total saponins (50 g). The crude extract of total saponins were preliminarily purified by macroporous resin D101 column chromatography (CC), eluted with $EtOH$-H_2O (60-100%, v/v), to afford five fractions (Frs. A-E). Fr. B was further eluted with CH_2Cl_2 - MeOH (10:3 to 10:5, v/v) over silica gel CC to obtain Frs. B_1-B_4. Among them, Frs. B_1, B_2, and B_4 were further purified by Sephadex LH-20 CC eluting with MeOH and Rp-18 silica gel CC eluting with MeOH-H_2O (20-80%) to obtain compounds **1** (173 mg), **2** (87 mg), and **5** (158 mg). Fr. B_3 was subjected to a MPLC with a stepwise gradient of MeOH-H_2O (60-100%) to afford compounds **3** (124 mg) and **4** (103 mg).

2.4. Biotransformation of monomer ginsenosides Rb_1, Rd, Re, Rg_1 and notoginsenoside R_1 by *A. tubingensis*

Biotransformation experiments were performed by adding ginsenosides to potato dextrose broth (PDB) liquid medium containing the fungus. Control experiments were performed in medium with only fungus. The biotransformation experiments were performed using PDB medium containing monomeric ginsenoside. The samples were incubated in a shaking incubator (160 rpm) at 26 °C for 21 days, respectively. The yield can be calculated using the following formula:

$$\text{Yield rate}(\%) = \frac{\text{amount of transformed product}}{\text{amount of transformed substrate}} \times 100\%$$

2.5. Preparation and preliminary purification of crude enzyme from *A. tubingensis*

To prepare a strain seed liquid, *A. tubingensis* was inoculated into PDB medium, and the mixture was cultured at 26 °C and 160 rpm for 5 days. To ensure consistent strain growth rate in subsequent cultures, equal amounts (10 mL) of seed fluid were added to 600 mL of

PDB medium and cultured for 7 days at 26°C and 160 rpm. Culture medium: potato extract powder (5.0 g/L), glucose (15.0 g/L), pH natural. After the fermentation solution was collected, $(NH_4)_2SO_4$ was added to the fermentation solution for protein precipitation. The addition amount of $(NH_4)_2SO_4$ was: 80g $(NH_4)_2SO_4$ was added to 100mL fermentation solution. The protein solution was dialyzed extensively and then freeze-dried according to a previously described method [27].

The enzyme was purified over a DEAE-cellulose DE-52 column (ϕ2 cm × 20 cm, Whatman). The enzymatic activity of fractions from this purification step were assayed using the substrate ginsenoside Rb_1. Ginsenoside Rb_1 hydrolysis was assessed via TLC, followed by collection and lyophilization of a portion of the hydrolyzed compound.

The crude enzyme that has been preliminarily purified was analyzed by polyacrylamide gel electrophoresis and determined by BCA protein concentration assay kit [28]. The crude enzyme, having undergone initial purification, underwent assay to determine its specific activity. The p-Nitrophenyl-β-D-glucopyranoside (pNPG) served as the substrate. The activity of β-glucosidase was quantified, defining one unit as the enzyme quantity yielding 1 μmol of p-nitrophenol (pNP) per minute in a 1 mL enzyme solution [29].

2.6. Crude enzyme basic characterization

2.6.1 Effect of pH on the activity and stability of β-glucosidase. The impact of pH on β-glucosidase activity was evaluated by utilizing pNPG as the substrate. The rates of reaction of the enzyme in sodium acetate buffers with pH values in the range of 4 to 8 were respectively determined following incubation at 50 °C for 20 min. The crude enzyme solution (30 mg/mL) was thoroughly mixed with a solution volume ratio of 2:1 for pNPG (1 mM) and incubated at 50°C for 20 min, followed by the addition of 0.5 M NaOH to halt the reaction and absorbances were read at 405 nm. The pH stability of the enzyme was assessed by incubating the β-glucosidase buffers with pH values from 4 to 8 for 0, 10, 20, 30, 40, and 50 min at 50 °C. The activities of β-glucosidase were measured by a colourimetric method using pNPG as substrate. Maximum activities were expressed relative to the maximum enzyme activity in this series of experiments. All tests were performed in triplicate.

2.6.2 Effect of temperature on the activity and stability of β-glucosidase. The effects of temperature on β-glucosidase activity was evaluated using pNPG as the substrate. For optimal temperature testing, the reaction mixture was determined in $NaC_2H_3O_2$ buffer (pH 5.0) a range of temperatures from 30 to 70 °C for 20 min. The crude enzyme solution (30 mg/mL) was thoroughly mixed with a solution volume ratio of 2:1 for pNPG (1 mM) and incubated at 50°C for 20 min, followed by the addition of 0.5 M NaOH to halt the reaction and absorbances were read at 405 nm. The enzyme's temperature stability was evaluated by incubating the β-glucosidase in the $NaC_2H_3O_2$ buffer (pH 5.0) for 0, 10, 20, 30, 40, and 50 min at 30, 40, 50, 55, 60 or 70°C. The activities of β-glucosidase were measured by a colourimetric method using pNPG as substrate. Maximum activities were expressed relative to the maximum enzyme activity in this series of experiments. All tests were performed in triplicate.

2.7. Analytical methods

The thin layer chromatography (TLC) was performed using $HSGF_{254}$ silica gel plates with chloroform: methanol: water (2:1:0.1, v/v/v) as the developing solvent. Spots on the TLC plates were identified by staining with 10% (v/v) H_2SO_4 in ethanol and heating at 110 °C for 2 min.

The identification of transformation products was achieved by liquid chromatography using an Agilent system (Grand Island, NY, USA) with an ACQUITY UPLC BEH C_{18}

column (1.7 μm, 2.1 × 50 mm, Waters, USA). The column was subjected to a gradient elution using water as mobile phase A and acetonitrile as mobile phase B. The elution gradient was programmed as follows: 0-3 min, 20% B; 3-5 min, B from 20% to 30%; 5-6 min, B from 30% to 35%; 6-8 min, B from 35% to 40%; 8-16 min, 40% B; 16-30 min, B form 40% to 45%; 30-45 min, B form 45% to 75%; and 45-55 min, B form 75% to 95%. The flow rate was 0.3 mL/min, samples were detected by absorption at 203 nm with an injection volume of 10 μL, and the column temperature was maintained at 30 °C.

3. Results and discussion

3.1. Characterization of *A. tubingensis*

We isolated a fungus from the soil in which *P. notoginseng* was growing, and we predicted that this fungus would be *A. tubingensis*. The ensuing colonies were found to be unevenly distributed throughout the plate, granular, and black in color (Fig 1A). When observed under a microscope, the cells were seen to have formed dark-brown to black conidia (Fig 1B). These features are consistent with those described in other studies of *A. tubingensis* [30,31]. When the sequence of the ITS rDNA gene of the strain was determined (supplemental file) and compared to sequences in the GenBank database, and this strain was identified as being in the genus *Aspergillus*, which sequence indicated a great deal of resemblance to the ITS rDNA gene from several strains of *A. tubingensis* (Fig 1C).

3.2. Isolation and purification of main saponins from *P. notoginseng* root

Isolation and purification of five main saponins compounds **1**–**5** from *P. notoginseng* root. Compounds **1**–**5** were identified as ginsenoside Rg_1 (**1**), notoginsenoside R_1 (**2**), ginsenoside Re (**3**), ginsenoside Rd (**4**) and ginsenoside Rb_1 (**5**), respectively. Their structures were elucidated by the comparison of their ^{13}C NMR data (S1 Table) with those of literature [32–34].
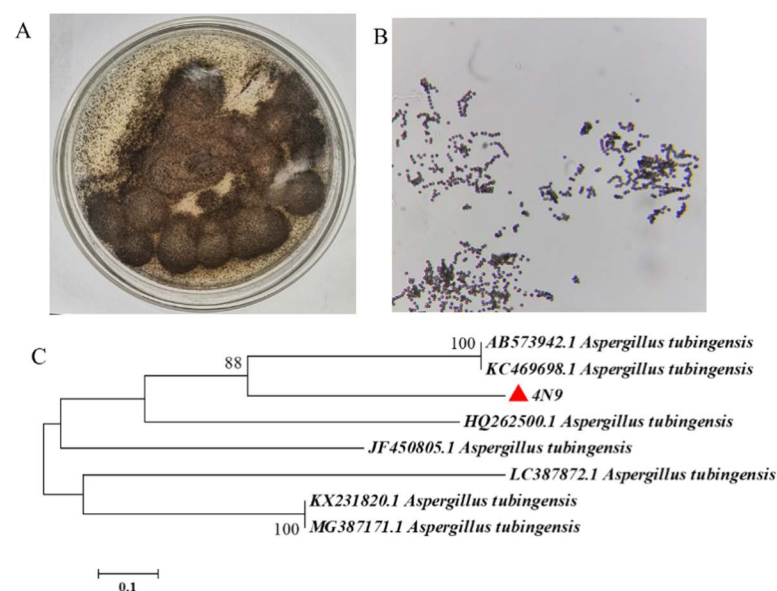

Fig 1. Morphology and phylogenetic tree of *A. tubingensis*. (A) Colony morphology diagram. (B) Conidiophore of *A. tubingensis*. (C) The phylogenetic tree based on ITS rDNA gene sequences of *A. tubingensis*.

https://doi.org/10.1371/journal.pone.0316279.g001

3.3. Biotransformation of major ginsenosides of *P. notoginseng* root by *A. tubingensis*

Major ginsenosides were subjected to biotransformation by *A. tubingensis*. The outcomes demonstrated *A. tubingensis* possesses a remarkable capacity to transformed the major ginsenosides. The presence of polar spots on TLC that are smaller than the polarity of the substrate suggests that minor saponins are forming (Fig 2A). Similarly, the results of the HPLC (Fig 2B) also indicated the production of several minor saponins.

Based on the chromatography findings, we proposed biotransformation pathways for the primary ginsenosides in *P. notoginseng* root. Each resultant product underwent additional scrutiny using LC-MS analysis. The Fig 3A depicts the proposed transformation pathway of ginsenoside Rb_1. The HPLC chromatogram (Fig 2B) revealed peaks attributed to small polar products, and these compounds were identified as Rd, (R/S)-Rg_3, Rk_1 and Rg_5. Accordingly, we suggest that Rb_1 was biotransformed into Rd, (R/S)-Rg_3, Rk_1 and Rg_5 by *A. tubingensis*. In addition, the results can also be illustrated in LC-MS analysis (S1A Fig).
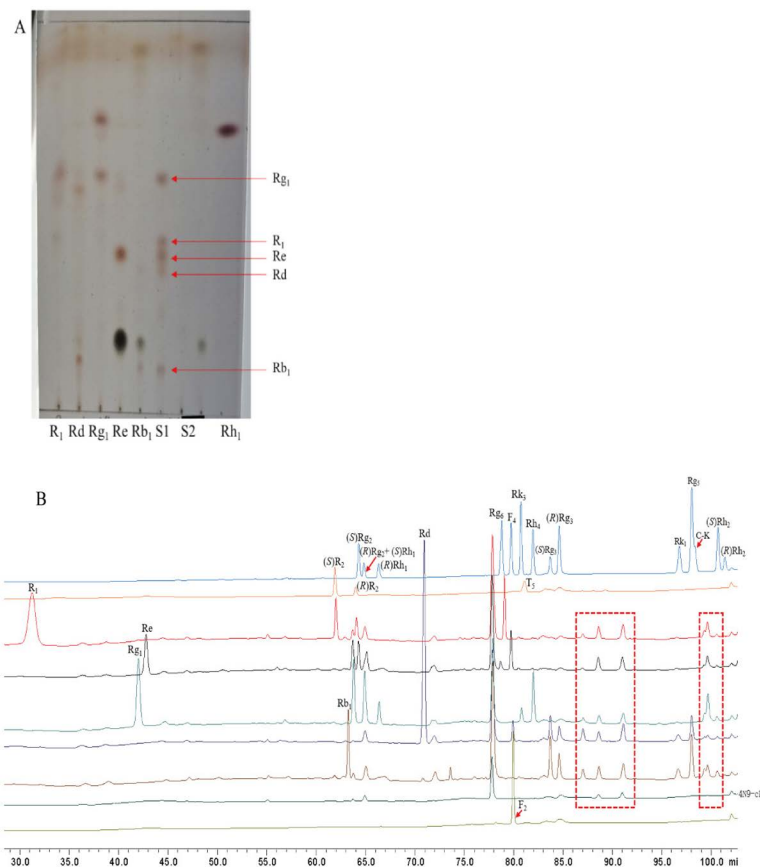

Fig 2. Analyses of the transformation products of five monomer ginsenosides by *A. tubingensis*. (A) TLC analysis of the transformation products by *A. tubingensis*. Rb_1, Rd, Re, Rg_1 and R_1: Representing their respective transformed products; S1: Authentic ginsenosides mixtures; S2: Metabolites of the *A. tubingensis*; (B) HPLC analysis of the transformation products by *A. tubingensis*. Rb_1, Rd, Re, Rg_1 and R_1: Representing their respective transformed products; 4N9-ck: Metabolites of the *A. tubingensis* (control, without added substrate); The red dashed boxes represent strain metabolites or unknown saponins.

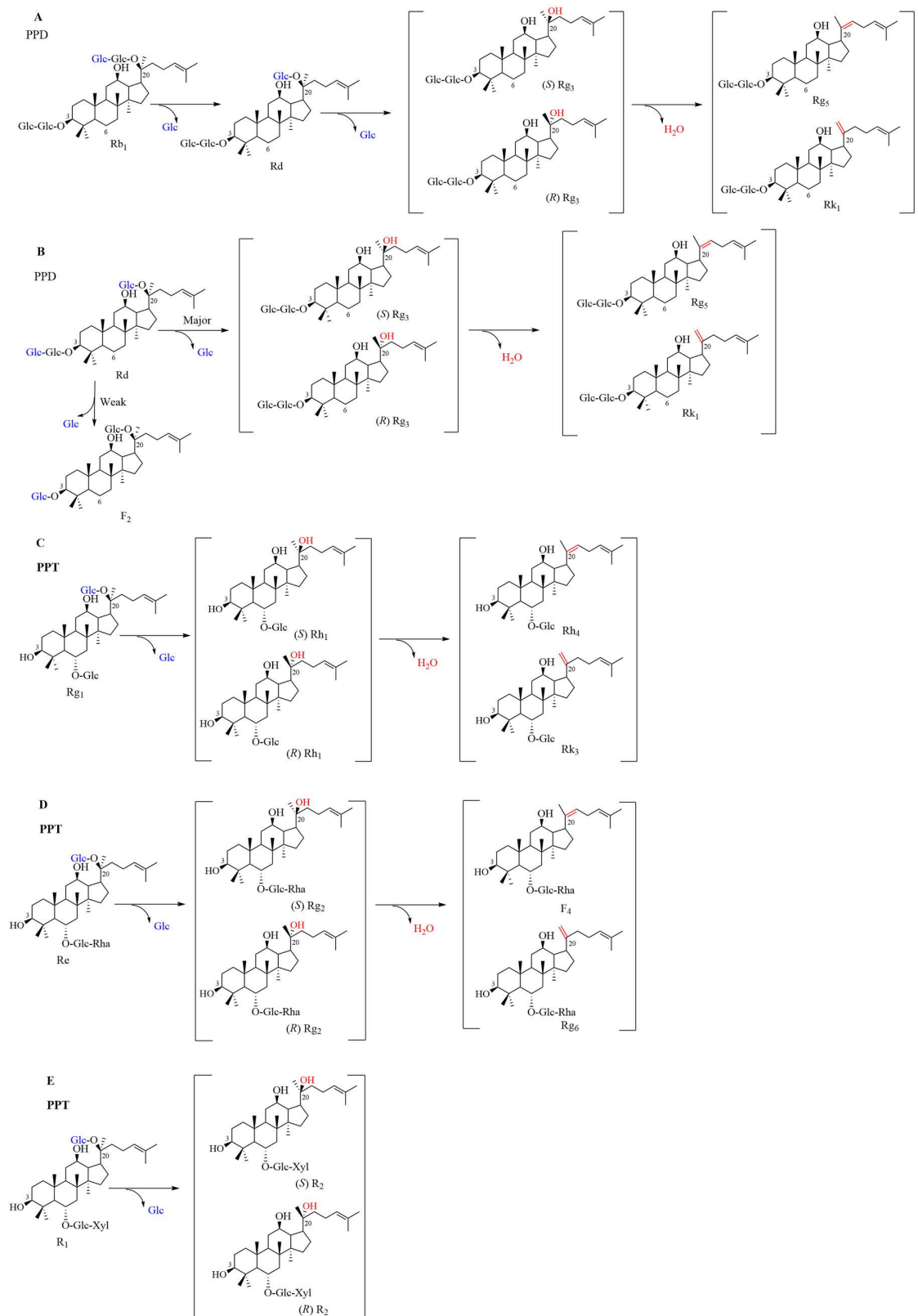**Fig 3. The proposed biotransformation pathways of five ginsenosides by *A. tubingensis*.** The proposed biotransformation pathways (A-E) of ginsenosides Rb$_1$, Rd, Rg$_1$, Re and notoginsenoside R$_1$ by *A. tubingensis*.

Based on the established attributes of saponin transformation routes, which typically hydrolyze single glycosyl fragments, we proposed that an enzyme from *A. tubingensis* hydrolyzed the 20-O-β-D-(1$\to$6)-glucopyranoside bond of Rb_1, yielding Rd. Subsequently, it catalyzed the hydrolysis of the inner 20-O-β-D-glucopyranoside moiety of Rd, resulting in the formation of (R/S)-Rg_3. Finally, it facilitated the dehydration process of the tertiary hydroxy group in (R/S)-Rg_3, generating Rk_1 and Rg_5. Therefore, the following was the transformation pathway of ginsenoside Rb_1: $Rb_1 \to Rd \to$ (R/S)-$Rg_3 \to Rk_1$ and Rg_5.

The proposed pathway of transformation of ginsenoside Rd is displayed in Fig 3B. Regarding the outcomes of the HPLC analysis (Fig 2B), we concluded that small polar compounds were present in the product, and these molecules were identified as F_2, (R/S)-Rg_3, Rk_1 and Rg_5. Therefore, we suggest that Rd was biotransformed into F_2, (R/S)-Rg_3, Rk_1 and Rg_5 by *A. tubingensis*. Similarly, the results can also be illustrated in LC-MS analysis (S1B Fig).

The enzyme from *A. tubingensis* can hydrolyzed the ginsenoside Rd to F_2 or Rd to (R/S)-Rg_3, and finally removed the tertiary hydroxy group in (R/S)-Rg_3 via dehydration to form Rk_1 and Rg_5. Therefore, the transformation routes of ginsenoside Rd were as follows: $Rd \to$ (R/S)-$Rg_3 \to Rk_1$ and Rg_5, and $Rd \to F_2$.

The Fig 3C shows the presumed transformation process of ginsenoside Rg_1. The small polar compounds present in the product as shown by HPLC analysis (Fig 2B) identified as (R/S)-Rh_1, Rk_3 and Rh_4. We suggest, then, that Rg_1 was biotransformed into (R/S)-Rh_1, Rk_3 and Rh_4 by *A. tubingensis*. Ultimately, the transformed outcomes of ginsenoside Rg_1 were evaluated by LC-MS (S1C Fig). According to the characteristics of saponin transformation pathways, we propose that the enzyme from *A. tubingensis* hydrolyzed the 20-O-β-D-glucopyranoside bond of Rg_1 to form Rh_1 and then removed the tertiary hydroxy group in (R/S)-Rh_1 via a dehydration reaction to form Rk_3 and Rh_4. Therefore, the transformation pathway of ginsenoside Rb_1 was proposed to be $Rg_1 \to$ (R/S)-$Rh_1 \to Rk_3$ and Rh_4. Similarly, the transformation pathway of ginsenoside Re and notoginsenoside R_1 were proposed to be $Re \to$ (R/S)-$Rg_2 \to F_4$ and Rg_6 (Fig 3D and S1D Figs) and $R_1 \to$ (R/S)-R_2 (Fig 3E and S1E Figs), respectively.

In HPLC analyses of the transformation pathways of ginsenosides Rb_1 and Rd, the peaks corresponding to the products of transformation of ginsenoside Rd were consistent with the retention time of standard ginsenoside F_2, while no peaks coeluting with standard F_2 were seen when analyzing the products of transformation of ginsenoside Rb_1. According to the hydrolysis pathways observed for other saponins, we predicted that ginsenoside F_2 would be hydrolyzed to C-K and that ginsenoside C-K would be hydrolyzed to Rh_2, but we did not observe C-K or Rh_2 in the transformation products; accordingly, we believe that the pathway of Rd hydrolysis to F_2 is relatively minor. The yield (%) of the minor ginsenosides from the major ginsenoside in *P. notoginseng* root upon transformation by *A. tubingensis* are shown in Table 1.

Table 1. The yields of the minor ginsenosides upon biotransformation of major ginsenosides from *P. notoginseng* root by *A. tubingensis*.

Substrates		Products	Yield (%)
PPD	Rb_1	(R/S)-Rg_3, Rk_1, Rg_5	23, 18, 8, 24
	Rd	F_2, (R/S)-Rg_3, Rk_1, Rg_5	5, 8, 7, 4, 9
PPT	Rg_1	(R/S)-Rh_1, Rk_3, Rh_4	19, 7, 4, 14
	Re	(R/S)-Rg_2, F_4, Rg_6	20, 17, 9, 23
	R_1	(R/S)-R_2	7, 12

https://doi.org/10.1371/journal.pone.0316279.t001

3.4. Purification and characterization of the biotransformation enzyme from *A. tubingensis*

In order to identify the enzyme responsible for the biotransformation, the supernatant was processed by anion exchange chromatography. Following the addition of the crude enzyme to the packed column, 0.05 M to 1.0 M KCl was progressively added to the column for elution. Eluted proteins were collected in 5.0 mL fractions.

Partial solutions of each fraction were mixed with ginsenoside Rb_1 and incubated for 48 h at 50 °C to measure the level of enzyme activity in each fraction. TLC is used to detect the transformation effect. Fractions 130 through 133, which contained proteins eluted with 0.4 M KCl, were found to hydrolyze ginsenoside Rb_1 into another ginsenoside with the highest activity (S2A Fig). When performing gel electrophoresis analysis, the molecular weight of the crude enzyme protein after preliminary purification is about 66 kDa (S2B Fig). The results of this crude enzyme protein is similar to those reported previously in the literature [4,35]. Considering the entire purification, the yield of enzyme was 3.4%, and the specific activity was increased by 4.8-fold (Table 2).

3.5. Effects of pH and temperature on the activity and stability of *β*-glucosidase from *A. tubingensis*

The Fig 4 illustrates the influence of temperature and pH on enzyme activity. The optimal pH of enzyme from *A. tubingensis* was pH 5, which was a relatively high activity (Fig 4A). The enzyme from *A. tubingensis* was found to be stable at a relatively wide range of pH values. As shown in Fig 4B, the enzyme from *A. tubingensis* was most stable in buffers with a pH range of 5 to 8, retaining approximately 70% of its activity. While the enzyme retained activity when incubated at pH 4, the enzyme activity was lower as compared to incubation at other pH levels.

The temperature at which the enzyme from *A. tubingensis* has the highest activity was found to be 50 and 55 °C (Fig 4C). Furthermore, the enzyme from *A. tubingensis* was also found to be stable at relatively high temperatures. As it shown in Fig 4D, when the enzyme was incubated at different temperatures and then assayed for activity, it was found to be particularly stable over the range of 30 °C to 70 °C, and the enzyme activity was maintained at approximately 70%. It has been reported that the optimal catalytic temperature of *β*-glucosidase from animals and plants is approximately 40°C, while the optimal catalytic temperature of *β*-glucosidase from microorganisms is higher, and the microorganismal enzyme is generally stable below 60 °C [36]. In this study, the enzyme from *A. tubingensis* retained activity at 70 °C, which implies that the enzyme is flexible with respect to temperature.

4. Conclusion

To improve the utilization of *P. notoginseng*, the PPD and PPT ginsenosides from *P. notoginseng* root were converted by the *A. tubingensis* to prepare minor ginsenosides. In this study, *A. tubingensis* was found to has the capable of convert major ginsenosides in *P. notoginseng* root (Rb_1, Rg_1, Re, and Rd and notoginsenoside R_1) into 15 minor ginsenosides. Besides, according to HPLC analyses of the transformation products of *P. notoginseng* ginsenosides converted by *A. tubingensis*, we found that six unknown compounds appeared during the conversion process. If we expand the scale of transformation, we may identify unknown components in the transformation products through isolation and identification methods. Therefore, it is possible to identify more minor ginsenosides and even new ginsenoside derivatives from the saponin conversion products.

Table 2. Purification of *β*-glucosidase from *A. tubingensis*.

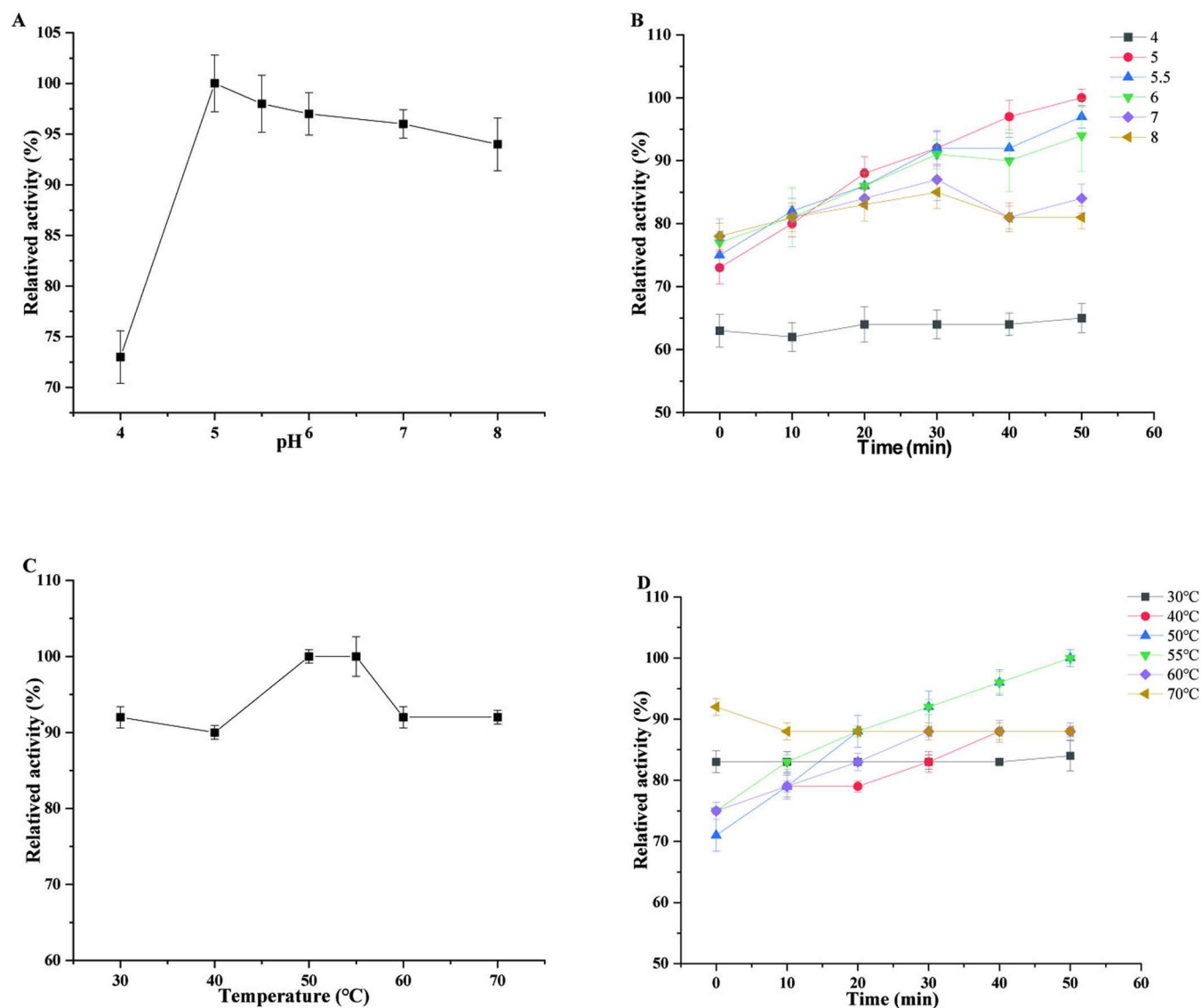| Step	Total protein (mg)	Total units (U)	Specific activity (U/mg)	Purification (-fold)	Yield (%)
Culture liquid	1534.0	21150.0	13.8	1	100
Precipitation of $(NH_4)_2SO_4$	65.28	2014.0	30.9	2.3	9.5
DEAE-cellulose	14.9	723.0	62.0	4.8	3.4

https://doi.org/10.1371/journal.pone.0316279.t002

Fig 4. Effects of pH and temperature on the activity and stability of *β*-glucosidase from *A. tubingensis*. (A) The effect of pH on the activity of *β*-glucosidase. (B) The effect of pH on the stability of *β*-glucosidase. (C) The effect of temperature on the activity of *β*-glucosidase. (D) The effect of temperature on the stability of *β*-glucosidase.

https://doi.org/10.1371/journal.pone.0316279.g004

The fungus can efficiently and selectively hydrolyze major ginsenosides in *P. notoginseng* root into minor ginsenosides involves three main types of reactions: hydrolysis, epimerization, and dehydration. In PPD-type ginsenosides, the major reaction involves the hydrolysis of the glucosyl moiety linked to C-20 and C-3. In PPT-type ginsenosides, we did not find evidence that the transformation reaction involves hydrolysis of the glucosyl moiety attached to C-6. Both types of saponins undergo isomerization and dehydration during the conversion process, resulting in the formation of 20(S,R)-epimers and double-bond isomers. Different from other *Aspergillus* species, it showed that *A. tubingensis* was able to form C-20(22) double-bond isomers by dehydration. For example, though the conversion reactions, the ginsenosides Rk_1, Rk_3, Rg_5 and Rg_6 can be formed, which are known to have antitumor activity.

The saponin transformation activity of *A. tubingensis* is notably robust, enabling it to concurrently convert both PPD and PPT ginsenosides from *P. notoginseng*. The resulting transformation products are quite diverse. Through meticulous analysis, we identified 15 minor ginsenosides among these transformation products. Furthermore, we have proposed the transformation pathways of ginsenosides, offering a robust theoretical foundation for the preparation and acquisition of minor ginsenosides. Our study provides a solid theoretical foundation and practical methods for the large-scale production of minor ginsenosides.

Supporting information

S1 Text. The sequencing of the ITS rDNA gene of *Aspergillus tubingensis* .
(DOCX)

S1 Fig. LC-MS analysis of transformation products of ginsenosides Rb_1, Rd, Rg_1, Re, notoginsenoside R_1 by *A. tubingensis* .
(TIFF)

S2 Fig. Enzyme characterization of *β*-ginsenosidase. (A) TLC analysis of ginsenosides Rb1 transformed by enzyme of different fractions. C: authentic ginsenosides; S1-S2: the fraction enzyme of ginsenoside-transformation activity; S3-5: the other fraction enzyme. (B) SDS-PAGE analysis of the purified *β*-glucosidase from *A.* tubingensis after protein staining with Coomassie Brilliant Blue solution. M: protein marker, a-f: purified enzyme.
(TIFF)

S3 Fig. ^{13}C spectrum (150MHz, C_5D_5N) of 1.
(TIF)

S4 Fig. ^{13}C spectrum (150MHz, C_5D_5N) of 2.
(TIF)

S5 Fig. ^{13}C spectrum (150MHz, C_5D_5N) of 3.
(TIF)

S6 Fig. ^{13}C spectrum (150MHz, C_5D_5N) of 4.
(TIF)

S7 Fig. ^{13}C spectrum (150MHz, C_5D_5N) of 5.
(TIF)

S1 Table. ^{3}C NMR data for compounds 1–5 in C_5D_5N.
(DOCX)

Author contributions

Conceptualization: Fei-Xing Li.

Data curation: Fei-Xing Li, Dong-Mei Lin.

Formal analysis: Fei-Xing Li, Dong-Mei Lin.

Funding acquisition: Xiao-Yan Yang.

Investigation: Fei-Xing Li.

Methodology: Fei-Xing Li.

Project administration: Xiao-Yan Yang.

Resources: Xiu-Ming Cui, Xiao-Yan Yang.

Software: Jin Yang.

Supervision: Xiao-Yan Yang.

Validation: Dong-Mei Lin.

Visualization: Fei-Xing Li, Xiao-Yan Yang.

Writing – original draft: Fei-Xing Li, Dong-Mei Lin.

Writing – review & editing: Fei-Xing Li, Xiao-Yan Yang.

References

1. Zhao Y-L, Zhang S-Q, Lu W-X, Shen S-Z, Wei L. Preparation of Panax notoginseng flower saponins enteric-coated sustained-release pellets and its pharmacokinetics and in vitro-in vivo correlation. J Drug Deliv Sci Tec. 2021;62:102321. https://doi.org/10.1016/j.jddst.2021.102321

2. Wang Z, Chen YY, Pan HJ, Wei L, Wang YH, Zeng CH. Saponin accumulation in flower buds of Panax notoginseng. Chin Herb Med. 2015;7:179–184.

3. Pei J, Xie J, Yin R, Zhao L, Ding G, Wang Z. Enzymatic transformation of ginsenoside Rb1 to ginsenoside 20(S)-Rg3 by GH3 β-glucosidase from Thermotoga thermarum DSM 5069T. J Mol Catal B: Enzym. 2015;113:104–9.

4. Liu C, Zuo K, Yu H, Sun C, Zhang T, Xu L, et al. Preparation of minor ginsenosides C-Mx and C-K from notoginseng leaf ginsenosides by a special ginsenosidase type-I. Process Biochemistry. 2015;50(12):2158–67. https://doi.org/10.1016/j.procbio.2015.10.011

5. Jia X-H, Wang C-Q, Liu J-H, Li X-W, Wang X, Shang M-Y, et al. Comparative studies of saponins in 1-3-year-old main roots, fibrous roots, and rhizomes of Panax notoginseng, and identification of different parts and growth-year samples. J Nat Med. 2013;67(2):339–49. https://doi.org/10.1007/s11418-012-0691-6 PMID: 22843418

6. Cao L, Wu H, Zhang H, Zhao Q, Yin X, Zheng D, et al. Highly efficient production of diverse rare ginsenosides using combinatorial biotechnology. Biotechnol Bioeng. 2020;117(6):1615–27. https://doi.org/10.1002/bit.27325 PMID: 32144753

7. Biswas T, Mathur AK, Mathur A. A literature update elucidating production of Panax ginsenosides with a special focus on strategies enriching the anti-neoplastic minor ginsenosides in ginseng preparations. Appl Microbiol Biotechnol. 2017;101(10):4009–32. https://doi.org/10.1007/s00253-017-8279-4 PMID: 28411325

8. Liang Y-Z, Guo M, Li Y-F, Shao L-J, Cui X-M, Yang X-Y. Highly Regioselective biotransformation of Protopanaxadiol-type and Protopanaxatriol-type ginsenosides in the underground parts of Panax notoginseng to 18 Minor Ginsenosides by talaromyces flavus. ACS Omega. 2022;7(17):14910–9. https://doi.org/10.1021/acsomega.2c00557 PMID: 35557696

9. Liu C, Jin Y, Yu H, Sun C, Gao P, Xiao Y, et al. Biotransformation pathway and kinetics of the hydrolysis of the 3-O- and 20-O-multi-glucosides of PPD-type ginsenosides by ginsenosidase type I. Process Biochem. 2014;49(5):813–20. https://doi.org/10.1016/j.procbio.2014.02.011

10. Yuan Z, Jiang H, Zhu X, Liu X, Li J. Ginsenoside Rg3 promotes cytotoxicity of Paclitaxel through inhibiting NF-κB signaling and regulating Bax/Bcl-2 expression on triple-negative breast cancer. Biomed Pharmacother. 2017;89227–32. https://doi.org/10.1016/j.biopha.2017.02.038 PMID: 28231544

11. Xue Q, Yu T, Wang Z, Fu X, Li X, Zou L, et al. Protective effect and mechanism of ginsenoside Rg2 on atherosclerosis. J Ginseng Res. 2023;47(2):237–45. https://doi.org/10.1016/j.jgr.2022.08.001 PMID: 36926610

12. Wu Y, Cui Y, Zhang T, Li W, Zhang M, Cheng J, et al. Transformation of ginsenoside Rh4 and its aglycone from the total saponins of stems and leaves of Panax ginseng by Aspergillus tubingensis. Phytochem Lett. 2017;27:123–8.

13. Xiao J, Chen D, Lin X-X, Peng S-F, Xiao M-F, Huang W-H, et al. Screening of drug metabolizing enzymes for the ginsenoside compound k in vitro: an efficient anti-cancer substance originating from panax ginseng. PLoS One. 2016;11(2):e0147183. https://doi.org/10.1371/journal.pone.0147183 PMID: 26845774

14. Mao Q, Zhang P-H, Wang Q, Li S-L. Ginsenoside F(2) induces apoptosis in humor gastric carci-noma cells through reactive oxygen species-mitochondria pathway and modulation of ASK-1/JNK signaling cascade in vitro and in vivo. Phytomedicine. 2014;21(4):515–22. https://doi.org/10.1016/j.phymed.2013.10.013 PMID: 24252332

15. Liu L, Wang H, Chai X, Meng Q, Jiang S, Zhao F. Advances in biocatalytic synthesis, pharmacologi-cal activities, pharmaceutical preparation and metabolism of ginsenoside Rh2. Mini Rev Med Chem. 2022;22(3):437–48. https://doi.org/10.2174/1389557521666210913114631 PMID: 34517798

16. Lu H, Yin H, Qu L, Ma X, Fu R, Fan D, et al. Ginsenoside Rk1 regulates glutamine metabolism in hepatocellular carcinoma through inhibition of the ERK/c-Myc pathway. Food Funct. 2022;13(7):3793–811. https://doi.org/10.1039/d1fo03728e PMID: 35316310

17. Qu L, Ma X, Fan D. Ginsenoside Rk3 suppresses hepatocellular carcinoma development through targeting the gut-liver axis. J Agric Food Chem. 2021;69(35):10121–37. https://doi.org/10.1021/acs.jafc.1c03279 PMID: 34415764

18. Liu Y, Fan D. The preparation of ginsenoside Rg5, its antitumor activity against breast cancer cells and its targeting of PI3K. Nutrients. 2020;12(1):246. https://doi.org/10.3390/nu12010246 PMID: 31963684

19. Chen B, Jia X-B. Apoptosis-inducing effect of ginsenoside Rg6 on human lymphocytoma JK cells. Molecules. 2013;18(7):8109–19. https://doi.org/10.3390/molecules18078109 PMID: 23839115

20. Jiang Y, Li W, Fan D. Biotransformation of ginsenoside Rb1 to ginsenoside CK by Strain XD101: a safe bioconversion strategy. Appl Biochem Biotechnol. 2021;193(7):2110–27. https://doi.org/10.1007/s12010-021-03485-0 PMID: 33629278

21. Kim S-A, Jeong E-B, Oh D-K. Complete Bioconversion of Protopanaxadiol-Type Ginsenosides to Compound K by Extracellular Enzymes from the Isolated Strain *Aspergillus tubingensis*. J Agric Food Chem. 2021;69(1):315–24. https://doi.org/10.1021/acs.jafc.0c07424 PMID: 33372793

22. Liu C-Y, Zhou R-X, Sun C-K, Jin Y-H, Yu H-S, Zhang T-Y, et al. Preparation of minor ginsenosides C-Mc, C-Y, F2, and C-K from American ginseng PPD-ginsenoside using special ginsenosidase type-I from *Aspergillus niger* g.848. J Ginseng Res. 2015;39(3):221–9. https://doi.org/10.1016/j.jgr.2014.12.003 PMID: 26199553

23. Kim YS, Kim DY, Kang DW, Park CS. Hydrolysis of the outer β-(1,2)-d-glucose linkage at the C-3 position of ginsenosides by a commercial β-galactosidase and its use in the production of minor ginsenosides. Biocatal Biotransfor. 2019;37:53–8.

24. Yu H, Liu Q, Zhang C, Lu M, Fu Y, Im W-T, et al. A new ginsenosidase from *Aspergillus* strain hydro-lyzing 20-O-multi-glycoside of PPD ginsenoside. Process Biochem. 2009;44(7):772–5. https://doi.org/10.1016/j.procbio.2009.02.005

25. Xiao Y, Liu C, Im W-T, Chen S, Zuo K, Yu H, et al. Dynamic changes of multi-notoginseng stem-leaf ginsenosides in reaction with ginsenosidase type-I. J Ginseng Res. 2019;43(2):186–95. https://doi.org/10.1016/j.jgr.2017.10.001 PMID: 30976159

26. Hu Y, Cui X, Zhang Z, Chen L, Zhang Y, Wang C, et al. Optimisation of Ethanol-Reflux Extraction of Saponins from Steamed Panax notoginseng by Response Surface Methodology and Evaluation of Hematopoiesis Effect. Molecules. 2018;23(5):1206. https://doi.org/10.3390/molecules23051206 PMID: 29772847

27. Kim D-W, Lee W-J, Gebru YA, Upadhyaya J, Ko S-R, Kim Y-H, et al. Production of Minor Ginsenosides C-K and C-Y from naturally occurring major ginsenosides using crude β-glucosidase preparation from submerged culture of fomitella fraxinea. Molecules. 2021;26(16):4820. https://doi.org/10.3390/molecules26164820 PMID: 34443407

28. Fan J, Zhang M, Ai Z, Huang J, Wang Y, Xiao S, et al. Highly regioselective hydrolysis of the glycosidic bonds in ginsenosides catalyzed by snailase. Process Biochemistry. 2021;103:114–22. https://doi.org/10.1016/j.procbio.2021.02.013

29. Shin K-C, Kim T-H, Choi J-H, Oh D-K. Complete biotransformation of protopanaxadiol-Type Ginsenosides to 20- O-β-glucopyranosyl-20(S)-protopanaxadiol using a novel and thermostable β-glucosidase. J Agric Food Chem. 2018;66(11):2822–9. https://doi.org/10.1021/acs.jafc.7b06108 PMID: 29468877

30. Xu C, Xu K, Yuan X-L, Ren G-W, Wang X-Q, Li W, et al. Characterization of diketopiperazine heterodimers as potential chemical markers for discrimination of two dominant black aspergilli, *Aspergillus niger* and *Aspergillus tubingensis*. Phytochemistry. 2020;176:112399. https://doi.org/10.1016/j.phytochem.2020.112399 PMID: 32408190

31. Mirhendi H, Zarei F, Motamedi M, Nouripour-Sisakht S. *Aspergillus* tubingensis and *Aspergillus* niger as the dominant black *Aspergillus*, use of simple PCR-RFLP for preliminary differentiation. J Mycol Med. 2016;26(1):9–16. https://doi.org/10.1016/j.mycmed.2015.12.004 PMID: 26852194

32. Teng RW, Li HZ, Chen JT, Wang DZ, He YN, Yang C, et al. Complete assignment of 1H and 13C NMR data for nine protopanaxatriol glycosides. Magn Reson Chem. 2002;40:483–488.

33. Kim DS, Chang YJ, Zedk U, Zhao P, Liu YQ, Yang CR. Dammarane saponins from Panax ginseng. Phytochemistry. 1995;40(5):1493–7. https://doi.org/10.1016/0031-9422(95)00218-v PMID: 8534407

34. Tanaka O, Han EC, Yamaguchi H, Matsuura H, Murakami T, Taniyama T, et al. Saponins of plants of panax species collected in Central Nepal, and their chemotaxonomical significance. III. Chem Pharm Bull (Tokyo). 2000;48(6):889–92. https://doi.org/10.1248/cpb.48.889 PMID: 10866157

35. Yu H, Zhang C, Lu M, Sun F, Fu Y, Jin F. Purification and characterization of new special ginsenosidase hydrolyzing multi-glycisides of protopanaxadiol ginsenosides, ginsenosidase type I. Chem Pharm Bull (Tokyo). 2007;55(2):231–5. https://doi.org/10.1248/cpb.55.231 PMID: 17268094

36. Zhang J, Zhao N, Xu J, Qi Y, Wei X, Fan M. Homology analysis of 35 β-glucosidases in Oenococcus oeni and biochemical characterization of a novel β-glucosidase BGL0224. Food Chem. 2021;334:127593. https://doi.org/10.1016/j.foodchem.2020.127593 PMID: 32711276