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Article

Microbial Transformation of Total Saponins from *Panax notoginseng* Flower by the Fungus *Talaromyces flavus*

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Abstract: Panax *notoginseng* is a traditional Chinese medicine, and saponins are its main active ingredients. The content of saponins in flower of *P. notoginseng* is the highest in the whole plant. Minor ginsenosides have stronger pharmacological activity than main ginsenosides, but its content in nature is very low. We can use extracted total saponins from *P. notoginseng* flower as the substrate to produce minor ginsenosides by biotransformation method. In this study, we used the fungus *Talaromyces flavus* and its crude enzymes to transform the extracted total saponins of *P. notoginseng* flower. Through the qualitative and quantitative analysis of the transformation substrates and products, it was found that minor ginsenosides Gyp-XVII, ginsenosides C-Mc$_1$, C-Mx$_1$, F$_2$, Rg$_3$, 20(*R*)-Rg$_3$, Rk$_1$ and Rg$_5$ were found in the products and the conversion rate of the major saponins in *P. notoginseng* flower ranged from 44.16 to 55.22%, the yields of the minor ginsenosides ranged from 0.05 to 3.24%. This study provided a good experimental idea, greatly increased the utilization rate of total saponins in *P. notoginseng* flower, and laid a foundation for promoting the wide application of minor saponins.

Keywords: *Panax notoginseng* flower; total saponins; microbial transformation; *Talaromyces flavus*; minor ginsenosides

1. Introduction

The genus of *Panax* belongs to the Araliaceae family, which has 17 species of the *P. genus*, including *P. ginseng*, *P. quinquefolius*, and *P. notoginseng*, etc [1]. Most members of this genus have medicinal properties and more than 150 ginsenosides have been identified and classified according to their structures, which can be divided into damarane, oktylon and oleanolic acid types [2,3]. *P. notoginseng* (Burk.) F. H. Chen is a traditional and valuable Chinese herbal medicine, mainly grown in Guangxi and Yunnan provinces in Southwest China. In *P. notoginseng*, dalmarane-type tetracyclic triterpene saponins can be further divided into propanaxadiol-type saponins (PPD, such as Rb$_1$, Rb$_2$, Rb$_3$, Rd and Rc) and propanaxtriol-type saponins (PPT, such as Re and Rg$_1$) according to the presence of a hydroxyl group attached to C-6. The kinds and contents of saponins in different parts of *P. notoginseng* are also different, and *P. notoginseng* flower (PNF) is the part with the highest content of saponins in *P. notoginseng* [4,5]. The saponins in PNF are mainly those containing more sugar groups. After oral administration, the major saponins need to be hydrolyzed by digestive enzymes and intestinal microorganisms before they can become more active and easily absorbed minor ginsenosides, but the efficiency of these conversions is very low [6].

Most studies have shown that the function of ginsenosides is closely related to the position, type and quantity of glycosidic bonds [7]. The main ginsenosides contain more sugar groups, which leads to their low pharmacological activity and is not easy to be absorbed by human body. Compared with the main ginsenosides, the minor ginsenosides have better pharmacological activities such as anti-

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hypertension, anti-aging, etc [8]. These minor saponins are produced mainly by deglycosylation, isomerization and dehydration [9,10]. The main ginsenosides can be hydrolyzed into minor ginsenosides by physical, chemical and microbial transformation. Compared with other methods, microbial conversion has the advantages of mild conversion conditions, product stability and pollution-free [11].

In order to prepare minor ginsenosides, many microorganisms capable of transforming ginsenosides have been discovered and applied. In particular, the relatively safe strain of *Aspergillus niger* is well known. *A. niger* JGL8 isolated from *Gynostemma pentaphyllum* can convert *Gynostemma pentaphyllum* saponins to ginsenoside F_2 through GypV→ Rd→ F_2 [12]. Ginsenoside Rb_1 can be transformed by extracellular enzymes produced by *A. niger* WU-16. The transformation pathway of Rb_1 is Rb_1→ F2, CK [13]. *A. Niger* XD101 and its crude enzymes screened from the planting soil of *P. notoginseng* also have the ability to hydrolyze ginsenoside Rb_1, and its conversion pathway is: Rb_1→ Rd→ F_2→ CK [14]. The results of all these studies indicate the feasibility of using microorganisms to carry out the transformation of major saponins for the preparation of minor saponins. Moreover, previous studies has shown that there are four main enzymes used in the enzymatic production of minor ginsenosides, these four enzymes hydrolyze glycosidic bonds at different positions on major ginsenosides [15-20].

In our previous study, an endophytic strain *T. flavus* was screened from the inter-root soil of *P. notoginseng* that could convert propanaxadiol-type (PPD) and propanaxtriol-type (PPT) ginsenosides in the underground part of *P. notoginseng* into 18 minor ginsenosides [21]. We found that some experiments were conducted by purchasing monomer major ginsenosides for biotransformation, which greatly increased the cost of experiments. In order to reduce the experimental cost and improve the utilization rate of PNF, the total saponins were extracted from PNF for conversion. The total saponins of PNF were analyzed by HPLC and it was found that it mainly contained ginsenosides Rb_1, Rc, Rb_2, Rb_3 and Rd in PNF. Through the biotransformation of extracted PNF total saponins, it was found that after 21d of PNF total saponins transformed by *T. flavus*, the main ginsenosides in PNF were transformed into ginsenosides Gyp-XVII, C-Mc_1, C-Mx_1, F_2, Rg_3, 20(*R*)-Rg_3, Rk_1 and Rg_5, and the conversion rates of the major saponins ranged from 44.16 to 55.22%, the yields of the minor saponins ranged from 0.05 to 3.24%. Additionally, the minor ginsenosides Gyp-XVII, C-Mc_1, C-Mx_1 and F_2 were found after the saponins of PNF were incubated for 10 days at pH 4.5 and 50°C with crude extracellular enzymes produced from *T. flavus*, and the conversion rates of the major saponins ranged from 19.92 to 36.21%. We also qualitative and quantitative analyzed the transformed substrates and products, which showed that *T. flavus* and its crude enzymes can effectively transform the saponins of PNF and produce minor ginsenosides.

2. Results and Discussion

2.1. Analysis of the results of PNF transformation by T. flavus

In our research, the total saponins of PNF were transformed by *T. flavus*. By HPLC analysis of PNF transformation products (Figure 1), it was found that chromatographic peaks such as 20(*S, R*)-Rg_3, Rk_1, Rg_5 were obviously present in the PNF transformation group compared with the substrate control group, and clearly unknown chromatographic peaks such as a and b.

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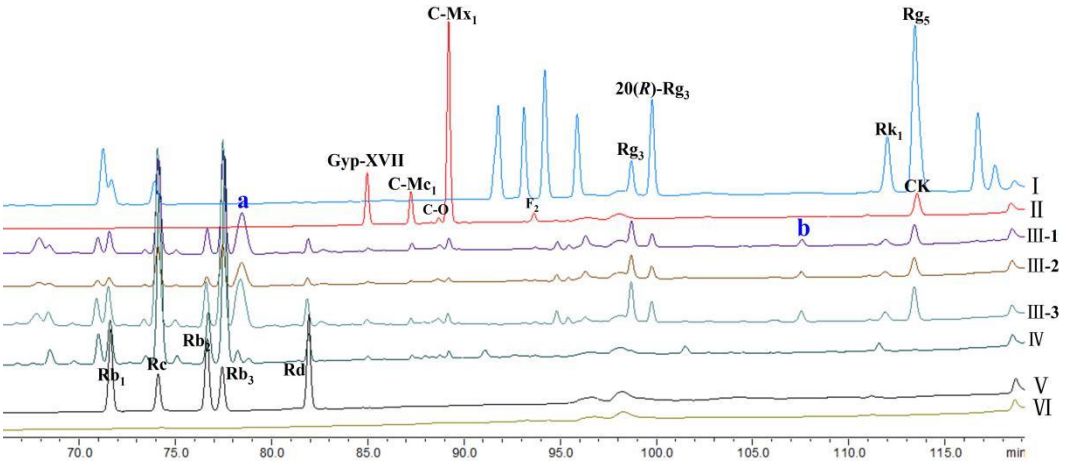

Figure 1. HPLC analysis of transformation products of PNF total saponins by *T. flavus*. I and II, Minor saponin standards; III, Transformation products of PNF (repeated 3 times); IV, PNF total saponins control; V, Main ginsenosides standards; VI, Fungal fermentation broth control.

Through the standard curve quantitative (Tables S1 and S2) and statistical analysis found that the contents of main saponins Rc and Rb_3 in the total saponins of PNF transformed group significantly decreased compared with the substrate control group, the amount of Rb_1 and Rb_2 extremely significantly decreased (Figure S1A). While, the content of minor ginsenosides Gyp-XVII, C-Mx_1, F_2, C-Mc_1 increased significantly. It is worth mentioning that minor ginsenosides 20 (*S, R*)-Rg_3, Rk_1, and Rg_5, which were not originally present, appeared in the conversion product (Figure S1B).

When the total saponins of PNF were transformed by *T. flavus*, the amount of total saponins of PNF in 5L medium was 0.712 g. The analysis and comparison of the PNF total saponins conversion levels were reflected by the average conversion of substrate and the yield of products, as Table 1.

Table 1. The transformation effect of PNF total saponins by *T. flavus*.

Substrates		**Products**	
Types	transformation rate (%)	Types	yield of products (%)
Rb_1	44.16	Gyp-XVII	0.18
Rc	55.22	C-Mc_1	0.05
Rb_2	48.63	C-Mx_1	0.14
Rb_3	47.25	F_2	0.07
		Rg_3	3.24
		20(*R*)-Rg_3	2.10
		Rk_1	0.58
		Rg_5	0.80

2.2. The total saponins of PNF were transformed by crude extracellular enzymes from T. flavus

After incubation of PNF saponins with crude enzyme for 10 days, the transformed products were extracted, dried, methanol redissolved and filtered by 0.45 μm filter membrane. HPLC analysis (Figure 2) showed that the peak areas of minor ginsenosides C-Mc_1, C-Mx_1, F_2 in the enzymatic conversion products of PNF total saponins were increased to a certain extent compared with the substrate control group.

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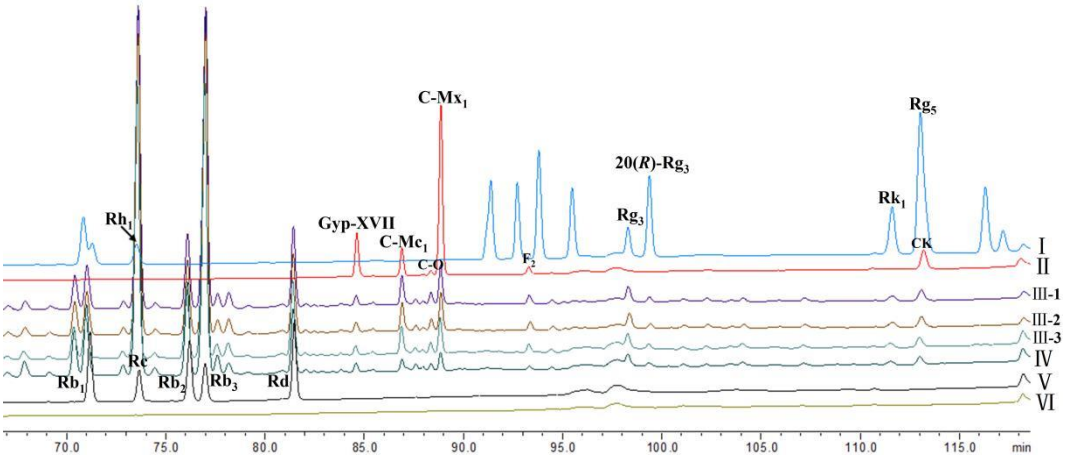

Figure 2. HPLC analysis of transformation products of PNF total saponins by extracellular crude enzymes of *T. flavus*. I and II, Minor saponin standards; III, Transformation products of PNF (repeated 3 times); IV, PNF control; V, Main ginsenosides standards; VI, Crude enzyme control.

Further quantitative and statistical analysis of saponins showed that, after conversion, ginsenosides Rb_2 and Rb_3 in PNF total saponins significantly decreased. Additionally, ginsenosides Rb_1 and Rc extremely significantly decreased, while and ginsenoside Rd significantly increased (Figure S2A). The contents of minor ginsenosides GypXVII, C-Mc_1, C-Mx_1 and F_2 increased extremely significantly (Figure S2B).

The total amount of PNF saponins in 1 mL buffer was 19.93 mg. The average conversion of main saponins and the yield of minor ginsenosides were analyzed as Table 2.

Table 2. The transformation effect of PNF total saponins by crude enzymes extracted from *T. flavus*.

Substrates		**Products**	
Types	transformation rate (%)	Types	yield of products (%)
Rb_1	36.21	Gyp-XVII	0.006
Rc	21.13	C-Mc_1	0.036
Rb_2	19.92	C-Mx_1	0.070
Rb_3	20.15	F_2	0.008

2.3. Discussion

In recent years, biotransformation and enzymatic catalysis have become popular methods for preparing minor ginsenosides, and many studies used these two methods to transform the main ginsenosides for the preparation of minor ginsenosides [22-24].

In our previous study, we found that *T. flavus* has the ability to convert monomer ginsenosides Rb_1, Rb_2, Rb_3, Rc, Rd and Rg_3 to minor ginsenosides by deglycosylation, isomerization and dehydration [21]. Therefore, we found the fungus *T. flavus* can hydrolyze the external glucose and internal glucose connected to C-20 in PPD ginsenosides, the external glucose connected to C-3 in PPT ginsenosides, and the internal glucose connected with C-20 in PPT ginsenosides. This suggests that it may possess multiple types of ginsenoside hydrolases and a strong capacity to hydrolyze ginsenosides, and these activities are similar to those described in the literature [25-27].

In this research, through a preliminary comparison of the fungal conversion effect and the enzyme conversion effect of the total saponins of self-extracted PNF, it was found that the types of minor ginsenosides produced by *T. flavus* transformation were more abundant and the content level was higher. There are two possible reasons for this result. On the one hand, the fungus can dynamically produce various saponin-degrading enzymes according to the actual level of saponins in the growing environment to promote the transformation. On the other hand, the combination of different substrates may promote the transformation effect. For example, when using Rb_1 as the only transformation substrate, multiple products such as Rg_3, Rh_2, CK and PPD can be obtained. However,

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when using Rb_1 and Re as the mixed transformation substrates, the content of Rg_3 in the products significantly increased [2]. In conclusion, it is feasible to produce many minor ginsenosides by the way of transformation PNF total saponins using *T. flavus*. However, the advantage of efficient transformation of enzyme protein is not well reflected in the transformation of self-extracted PNF total saponins. If the main saponins in self-extracted PNF total saponins were separated and purified one by one by using preparative liquid chromatography, the monomer saponins with a certain purity were prepared and then incubated with the crude enzyme, which may increase the probability of the enzyme protein binding to the suitable substrate, rapid and targeted hydrolysis of main ginsenosides to minor ginsenosides.

A rough comparison with the literature reports on the transformation of ginsenoside extracts by other microorganisms (Table S3) showed that the average substrate conversion rate of *T. flavus* in transforming ginsenoside extracts was generally in the middle level, however, as far as the abundance of transformation products is concerned, the minor ginsenoside types obtained in this study occupy an absolute advantage.

In order to unify with the indexes in the literature, when comparing the average conversion level of minor ginsenosides in the transformed products, only some of the minor ginsenosides in the transformation products of *T. flavus* were selected for comparison. The results showed that the average transfer level of minor ginsenoside Rg_3 produced by *T. flavus* conversion of PNF total saponins was in the top position (2/11), that is, ranked the second among the 11 conversion results under the same comparison item. On the whole, the *T. flavus* showed high substrate conversion rate in the process of transforming ginsenoside crude extracts to enrich minor ginsenosides, and the resulting minor ginsenosides were also abundant, which had a good application prospect.

3. Materials and Methods

3.1. Materials

T. flavus was obtained by the laboratory in the early stage, and the total saponins of PNF were extracted by the laboratory and qualitatively and quantitatively. Ginsenosides Rb_1, Rb_2, Rb_3, Rd, Rc, Rg_3, F_2, 20(*R*)-Rg_3, Rk_1, Rg_5, CK, Rh_1, Rd_2 C-Mx_1, C-Mc_1, and Gypenoside XVII (HPLC > 98%) were purchased from Sichuan Shengli Biotechnology Co., LTD. The solvents for HPLC, methanol and acetonitrile, were purchased from Sigma-Aldrich Co. (St. Louis, MO, USA). Other analytical grade reagents were purchased from commercial sources.

3.2. HPLC procedure and methodology

The elution procedures as follows: 0-25 min (20 % B), 25-73 min (20-38 % B), 73-78 min (38-46 % B), 78-80 min (46-49 % B), 80-90 min (49-56 % B), 90-92 min (56-62 % B), 92-102 min (62-75% B), 102-105 min (75-100 % B). The LC-20AB High-performance liquid chromatography was used for liquid chromatography. The chromatographic column was C_{18} column (5 μm, 4.6 × 250 mm), the detection wavelength was 203 nm, the column temperature was 35°C, the volume flow rate was 1.0 mL/min, and the injection volume was 25 μL, the mobile phase is water (A)-acetonitrile (B). For the standard curve, the lowest limit of detection (LOD) and the lowest limit of quantification (LOQ) were calculated, respectively, as well as RSD in precision trials, repeatability trials, and stability trials [27].

3.3. Transformation of P. notoginseng saponins by T. flavus

The PNF concentration was 0.05 mg/mL. The total saponins of PNF were transformed by *T. flavus* for 21 days as the experimental group, and PNF as the blank control group. In order to facilitate the comparison and significance analysis between groups, the total amount of saponin (mg) in the transformed product extract was measured as the main index. The average conversion rate of main saponins is shown in Formula (1), and the average yield of minor saponins is shown in Formula (2):

$$\text{Conversion rate} = \frac{\text{Pre-conversion content (mg)} - \text{Post-conversion content(mg)}}{\text{Pre-conversion content(mg)}} * 100\% \quad \ldots (1)$$

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$$\text{Yield rate} = \frac{\text{Content of transformed products(mg)}}{\text{Content of substrate before transformation(mg)}} * 100\% \quad \ldots (2)$$

SPSS 23.0 software was used for statistical analysis, and T-test was used for significance analysis between the two groups of data.

3.4. Transformation of PNF by crude extracellular enzymes

The extracelluar crude enzyme was extracted by ammonium salt precipitation method [21]. The amount of PNF and crude enzyme was 0.02 mg and 2.00 mg, respectively, and were incubated at pH 4.5, temperature 50°C and total reaction volume 1 mL for 10 days.

4. Conclusion

This study was used *T. flavus* and its crude extracellular enzymes to transform total saponins of PNF to minor ginsenosides. Because the crude extracellular enzyme and the total saponins extracted by our laboratory were prepared by our own, their purity can be further improved to obtain better transformation effect. In conclusion, this study further verified that *T. flavus* could use the total saponins of PNF as substrate, the main saponins in PNF were converted into minor saponins Gyp-XVII, C-Mc$_1$, C-Mx$_1$, F$_2$, Rg$_3$, 20(*R*)-Rg$_3$, Rk$_1$ and Rg$_5$. In this study, the utilization rate of *P. notoginseng* was increased to a certain extent, and the production of minor saponins by self-extracting PNF total saponins was expanded. These findings can be applied to the extraction of saponins from other parts of *P. notoginseng* and the production of minor saponins with self-extracted saponins. It can lay a foundation for the subsequent construction of genetic engineering strains and the large-scale production of minor saponins.

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