Transformation of Panax notoginseng saponins by steaming and Trichoderma longibrachiatum

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ABSTRACT

Panax notoginseng has been used for medicinal purposes in China for many years. Saponins are believed to be the major bioactive ingredients in P. notoginseng. Two different processes, steaming and biotransformation, were used to study the transformations of saponins in P. notoginseng. During an 8-h steaming process, the ginsenosides Rb1, Rd, Rg1, Re and notoginsenoside R1, decreased to 1.07 mg/g dry weight (DW), 0.91, 0.64, 0 and 0 mg/g DW, respectively. Meanwhile, ginsenoside 20(S)-Rg3, 20(S)-Rh1, F2 and compound K significantly increased to 5.85, 6.10, 0.81 and 6.62 mg/g DW, respectively. On the other hand, one fungus was isolated from the root of P. notoginseng, which could transform ginsenoside Rb1 to ginsenoside Rd specifically. The fungus was identified as Trichoderma longibrachiatum species based on sequence analysis of the rRNA internal transcribed spacers region. The results implied a prospective feasibility for setting up different processing techniques to improve the quality of P. notoginseng and add its value.

KEYWORDS

Panax notoginseng; ginsenoside Rb1; ginsenoside Rd; biotransformation

Introduction

Panax notoginseng (Chinese ginseng) is one of the Chinese traditional medicinal plants, which belongs to Panax genus, Araliaceae family and has been used by ethnic people in Southwestern China for treatment of cardiovascular diseases, inflammation, body pain and trauma for more than 400 years. It was included as a dietary supplement by the US Dietary Supplement Health and Education Act in 1994.[1] Traditionally, raw notoginseng is used as trauma hemostatic and cardiovascular medicine, whereas the processed form is used as tonic because of its blood cell increasing functions. In the past 20 years, many chemical and pharmacological studies on P. notoginseng have been carried out.[2–4] Triterpenoid saponins, including ginsenosides, notoginsenosides and gypenosides, are considered to be the main bioactive compounds. So far, more than 70 saponins have been isolated from P. notoginseng, among which ginsenosides Rb1, Rg1, Re and notoginsenoside R1 are the major components, whereas the others have low contents.[5]

Various studies have focused on the pharmacological activities of the minor ginsenosides, as their activities were found to be more valuable than those of the major ginsenosides.[6–8] The minor ginsenosides, including ginsenosides F1, F2, Rg3, compound K (C-K) and Rh1, can be produced by hydrolysis of glycosides from the major ginsenosides Rg1, Re, Rb1 and notoginsenoside R1. Therefore, some reports have aimed to convert major ginsenosides to minor ginsenosides with higher activities.[9–11] Several transformation methods have been used to achieve the aim,[11,12] including mild acid hydrolysis,[13] enzymatic conversion,[14] and microbial conversion,[15] but the chemical methods also bring side reactions such as epimerization, hydration and hydroxylation.[16]

Because of the relative backward processing technology, the traditional Chinese medicine was used mainly for rough processing. It would be significant to make full use of the existing resources and add their value by means of utilizing new and advanced processing technologies.

In this study, the two processing methods of steaming and microbial transformation of P. notoginseng were compared and the dynamic curves of saponin transformation were investigated. One fungus was isolated from the root of P. notoginseng, which could transform ginsenoside Rb1 to ginsenoside Rd specifically. The possible transformation pathways of saponins in the processes were also discussed.

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Materials and methods

Materials

The standards of ginsenosides were obtained from the National Institutes for Food and Drug Control, China. Their chemical structures are shown in Figure 1. Acetonitrile was purchased from Merck Darmstadt, Germany and methanol was purchased from Tianjin Chemical Ltd., China. Raw roots of *P. notoginseng* were purchased from Miaoxiang Ltd., Wenshan country, Yunnan province, China and comminuted to 60-mesh size particles with electric grinder (Beijing Zhongxingweiye Instrument Co. Ltd., China) and sieve.

Fungal cultures

WYC2012 was one of the microorganism strains isolated from raw roots of *P. notoginseng*. We cultivated it on potato dextrose agar (PDA) containing 2% (w/v) sucrose, 2% (w/v) agar and 20% (v/v) potato extract. It was cultured on PDA medium at 23 °C for five days and stored at 4 °C until further use.

Fungal spore suspensions were prepared by washing five-day-old culture slants with sterile 0.9% NaCl and shaking at 100 rpm for 20 min. Spores were counted with a haemocytometer and adjusted to approximately 10⁷ spores per mL.
Steaming of notoginseng

Notoginseng root (dry) was steamed at 120 °C for 0.5, 1, 1.5, 2, 3, 4, 6 and 8 h. The raw notoginseng was used as control. The raw and steamed samples were frozen for 2 h before lyophilization (Shanghai Pudong Freeze Dryer Equipment Co. Ltd., China). The obtained ginsenoside contents were expressed as mg/g of dry weight (DW).

Biotransformation of ginsenosides

Erlenmeyer flasks (100 mL) containing 20 mL of sterilized medium, with an initial pH of 5.0 were inoculated with P. notoginseng or monomer ginsenoside (ginsenoside Rb1) was added to a final concentration of 10 mg/mL or 1 mg/mL (the latter was used in the present study) to initiate biotransformation. The sterilized raw notoginseng, which proceeded the biotransformation without the WYC2012 fungal strain, was used as control. The fermentation times were 24, 36, 48, 60, 72, 84, 96, 108 and 120 h. Three flasks were run under the same conditions and the data represented were the mean values with standard deviations from three independent samples. The obtained ginsenoside contents were expressed as mg/g of DW.

High performance liquid chromatography (HPLC) analysis

The raw and steamed samples and biotransformed mixtures were extracted with n-butanol saturated with H2O, evaporated under vacuum (Shanghai Jinghong Laboratory Equipment Co. Ltd., China), and the residues were dissolved in methanol for the HPLC analysis.

HPLC analysis was performed on a Dionex Ultimate 3000 liquid chromatograph (Sunnyvale, CA, USA) equipped with a quaternary pump, an automatic injector and a photodiode array detector 3000. The reversed-phase column used was a Waters Symmetry C-18 (5 μm, 4.6 mm × 250 mm) column. The gradient elution system consisted of water (A) and acetonitrile (B). Separation was achieved by using the following gradient: 0–30 min: 20% B, 30–60 min: 20%–45% B, 60–78 min: 45%–75% B, 78–80 min: 75%–80% B. The column temperature was set at 30 °C. The flow rate was 1 mL/min and the injection volume was 10 μL. The UV detection wavelength was set at 203 nm. The compounds quantification was done via standard curves, which were made by using reference compounds.

Molecular methods

The genomic DNA of the strain WYC2012 was extracted and purified by the E.Z.N.A. Fungal DNA Kit (Omega Bio-Tek Inc., USA). The internal transcribed spacer (ITS) rRNA gene sequences of the strain WYC2012 was sequenced by the Shanghai Majorbio Bio-Pharm Technology Co. Ltd., China. The ITS rRNA gene sequences of the related taxa were obtained from GenBank. A phylogenetic tree was constructed using the neighbour-joining method through the MEGA 5.05 programme. A bootstrap analysis with 1000 replicates was also conducted to obtain confidence levels for the branches. ITS1-5.8S-ITS2 rRNA gene sequences of the strain WYC2012 were aligned with those of the type strains found to have the closest taxonomic relationships.

Two oligonucleotide primers, forward primer 5’-GGAGTAAAAGTCAACAGG-3’ and reverse primer 5’-TCCTCCGCTTTATGATAGC-3’, were used for the polymerase chain reaction (Applied Biosystems, USA) to amplify the ITS rRNA gene. The amplification procedure was: 95 °C for 4 min; then 30 cycles at 94 °C for 45 s, 53 °C for 45 s and 72 °C for 50 s, with a final extension at 72 °C for 8 min.

Statistical analysis

A one-way analysis of variance (ANOVA) was employed to determine whether the results had statistical significance. In some cases, Student’s t-test was used for comparing two groups. Both statistical analyses were processed by SPSS (Statistical Product and Service Solutions) software. The level of statistical significance was set at p < 0.05.

Results and discussion

Effect of steaming on notoginseng constituents

The HPLC chromatograms of raw P. notoginseng, steamed P. notoginseng and microbially transformed P. notoginseng are shown in Figure 2. The contents of ginsenosides in the raw and steamed samples are shown in Table 1. The results indicated a distinct difference in saponin composition between the raw and steamed P. notoginseng. During the steaming process, ginsenosides Rb1, Rd, Rg1, Re and notoginsenoside R1, the five main saponin constituents in the raw notoginseng, decreased gradually, and reached 1.07, 0.91, 0.64, 0 and 0 mg/g DW, respectively during the 8-h steaming process, whereas some new saponins were formed (Figure 3).

In the process of steaming, the transformation of saponins began at an early stage. For instance, decreases of Rg1, Rb1, Rd, Re and R1 could be observed evidently after 2 h of steaming (Figure 3(A)). Then, the extent of transformation (both degradation and formation) of saponins increased steadily during the steaming process. After 4 h of steaming, Re was difficult to be detected in
the chromatogram; Rg1, Rb1, Rd and R1 were at very low concentrations; ginsenoside 20(S)-Rg3, 20(S)-Rh1 and F2, which were trace saponins in raw notoginseng, significantly increased to 5.85, 6.10, 0.81 mg/g DW, respectively; C-K reached 6.69 mg/g DW, which could not be detected in raw P. notoginseng (Figure 3(B)). The peak areas of six ginsenosides, 20(R)-Rh1, 20(R)-Rg3, Rk3, Rh4, Rk1 and Rg5, were significantly increased as compared to the raw sample (Figure 2(B)).

The glycosyl group is prone to being hydrolyzed in dammarane-type tetracyclic triterpenoid glycosides of notoginseng. Some reactions, including rearrangement, reduction and oxidation, can occur at the side chain to produce diverse products.[17,18] The mechanism of saponin transformations in the steaming process could be extrapolated from the chemical structures of saponins, especially the changes in their sugar moiety. During the steaming, the C-20 glucosyl group of ginsenoside Rb1 was hydrolyzed to yield ginsenoside Rd. The hydrolysis of the xylosyl residue attached to C-6 of ginsenoside R1 and the hydrolysis of the rhamnosyl residue at C-6 of ginsenoside Re formed ginsenoside Rg1. Rg1 and

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**Figure 2.** HPLC chromatograms of raw P. notoginseng (A), steamed P. notoginseng at 120 °C for 4 h (B) and biotransformed P. notoginseng with T. longibrachiatum for 72 h (C). Note: The chromatographic peaks were identified by comparing the retention time with that of each reference compound; Notoginsenoside R1 (1); ginsenoside Rg1 (2); ginsenoside Re (3); ginsenoside Rb1 (4); ginsenoside 20(S)-Rh1 (5); ginsenoside Rd (6); ginsenoside F2 (7); ginsenoside 20(S)-Rg3 (8); ginsenoside C-K (9); ginsenoside 20(R)-Rh1 (10); ginsenoside Rk3 (11); ginsenoside Rh4 (12); ginsenoside 20(R)-Rg3 (13); ginsenoside Rk1 (14); ginsenoside Rg5 (15).
Rd were likely to be the parent compounds of newly formed saponins. Further hydrolysis of the glucosyl group at C-20 of Rg1 yielded Rh1, which then formed Rh4 and Rk3 through dehydration at C-20. Similarly, the hydrolyzation of the glucosyl groups at C-3 and C-20 of Rd produced F2 and Rg3, respectively. Ginsenoside F2 was hydrolyzed at the glucosyl group of C-3 to yield C-K. The dehydration of Rg3 at C-20 yielded Rg5 and Rk1 (Figure 4).

**Biotransformation of ginsenosides by WYC2012**

The identity of the confirmed saponins following biotransformation is shown in Figure 2(C) and Table 2. Ginsenoside Rb1 (12.03 mg/g DW) and Rg1 (14.95 mg/g DW), the main constituents in raw notoginseng, were transformed significantly by the fungal fermentation. After fermentation for 72 and 120 h, Rb1 and Rg1 decreased with 96.6% and with 28.1%, respectively.

During a fermentation time from 0 to 72 h, the protopanaxatriol groups of R1, Rg1, Re and 20(S)-Rh1 changed little, but the protopanaxadiol group of Rb1 and Rd changed significantly. The level of the ginsenoside Rd significantly increased from 3.54 to 15.40 mg/g DW, and the level of ginsenoside Rb1 decreased from 12.03 to 0.41 mg/g DW after 72 h of fermentation.

Some reports have demonstrated that ginsenoside Rb1 is deglycosylated by intestinal bacteria after oral administration.[19,20] The deglycosylated aglycone metabolites, such as compound K, Rd and Rg3 have a wide range of pharmacological properties. Therefore, knowledge, regarding the biotransformation of major ginsenosides to their respective deglycosylated metabolites, is of great importance.

In order to confirm that ginsenoside Rd was derived from ginsenoside Rb1, monomer ginsenoside Rb1 was added to the medium for biotransformation. The conversion yield of ginsenoside Rb1 by WYC2012 was
confirmed quantitatively by HPLC analysis. As shown in Figure 5, almost all ginsenoside Rb1 was transformed to ginsenoside Rd. Thus, WYC2012 transformed 1.0 mg/mL ginsenoside Rb1 to 0.791 mg/mL ginsenoside Rd, corresponding to a molar conversion yield of 92.6%.

Theoretically, four glucose moieties attached to the ginsenoside Rb1 could be available for hydrolysis by WYC2012, namely, the outer and inner glucose moieties attached at positions C-3 and C-20. Based on an analysis of the hydrolysis products of ginsenoside Rb1, WYC2012 hydrolyzed the outer glucose moiety attached to the C-20 position for ginsenoside Rd production. Furthermore, the inner glucose moiety attached to the C-3 position of ginsenoside Rb1 was not hydrolyzed.

**Phylogenetic study**

The phylogenetic tree constructed during the study is shown in Figure 6. Strain WYC2012, which was calculated to belong to the *Trichoderma* genus, had the highest
degree of ITS rRNA gene sequences similarity with the *T. longibrachiatum* strain DAOM 167674 (EU280099) (100%) and *T. longibrachiatum* strain DAOM 231259 (EU401562) (99%). Therefore, the strain WYC2012 was classified as *T. longibrachiatum*. As determined by taxonomic evaluation, strain showing less than 1% difference in its ITS rRNA gene sequence with the corresponding type stains was assumed to belong to the same species as the type stains.

**Conclusions**

The biotransformation of major ginsenosides to their respective deglycosylated metabolites is of great importance and several kinds of microbes have been used in such researches. In the present study, *T. longibrachiatum* WYC2012 could convert ginsenoside Rb1 to ginsenoside Rd with high selectivity, and the molar conversion yield reached 92.6%. The specificity of the biotransformation (Rb1→Rd), which can produce ginsenoside Rd efficiently, has not been reported before. In conclusion, two different processes, steaming and biotransformation, were used to study the transformations of saponins in *P. notoginseng*. The result implies a prospective feasibility for setting up different processing techniques to improve the quality of *P. notoginseng* and add its value. On the other hand, processed *P. notoginseng* will provide a potential access to certain effective saponin components, such as Rh1, Rg3, compound K, Rd which are in low contents or absent in plants.

**Disclosure statement**

The authors declare that they have no conflict of interests.

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**Compliance with Ethics Requirements**

This article does not contain any studies with human or animal subjects.

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