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SHORT COMMUNICATION

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Preparation of przewalskinic acid A from salvianolic acid B using a crude enzyme from an *Aspergillus oryzae* strain

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Abstract Przewalskinic acid A is a rare, water-soluble, and highly biologically active ingredient found, thus far, only in the *Salvia przewalskii* Maxim herb; however, the content in *S. przewalskii* herb is very low. In order to obtain useful quantities of przewalskinic acid A, the biotransformatin of salvianolic acid B from *Salvia miltiorrhiza* root (danshen in Chinese) into przewalskinic acid A was studied using a crude enzyme produced from *Aspergillus oryzae* D30s strain. The crude enzyme from the *A. oryzae* strain hydrolyzed salvianolic acid B into przewalskinic acid A and danshensu. The preparation afforded 31.3 g przewalskinic acid A (91.0 % purity) and 13.1 g danshensu (95 % purity) from 75 g salvianolic acid B. The preparation of przewalskinic acid A was therefore very successful with a yield of over 86 %, but the yield of danshensu was only 33 %. The product przewalskinic acid A was identified using ultra-performance liquid chromatography–mass spectrometry (UPLC–MS) and NMR.

Keywords Przewalskinic acid A · Salvianolic acid B · *Salvia miltiorrhiza* root · Biotransformation · *Aspergillus oryzae*

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Introduction

The dried root of *Salvia miltiorrhiza* Bunge (danshen in Chinese) is one of the most popular herbal traditional medicines in China and Asian countries [22, 25], and has been widely used for the treatment of many diseases such as coronary artery diseases, angina pectoris, myocardial infarction, cerebrovascular diseases [8, 25], neurasthenic insomnia [17], hepatocirrhosis [12, 19], chronic renal failure, and dysmenorrhea [5, 16]. The active ingredients of *S. miltiorrhiza* are classified into two groups, i.e., hydrophilic phenolic acids (water-soluble ingredients) and lipophilic diterpenoid quinones. Salvianolic acid B, as the major water-soluble ingredient of *S. miltiorrhiza* (containing over 3 %) [11, 13, 14], is mainly responsible for the therapeutic effects of *S. miltiorrhiza* medicine.

However, the ingredients of herbs (natural products) usually have low activity and are absorbed and utilized directly by the human body with difficultly. After oral administration, the ingredients are converted to easily absorbed and more active compounds by intestinal bacteria and digestive enzymes in the gastrointestinal tract, and thereafter absorbed by the body; but this transformation is often not very efficient [6, 7, 9]. After oral administration, salvianolic acid B was possibly transformed into more active and easily absorbed compounds, such as danshensu [3-(3,4-dihydroxyphenyl)lactic acid] and przewalskinic

acid A, in the human digestive system to be absorbed by the body. There have been many studies about danshensu such as the chemical structure, preparation, and biological activities [8, 10, 20, 21], but little research has been done on przewalskinic acid A [1, 13, 15].

The rare przewalskinic acid A is a small-molecule phenolic acid thus far only found in *Salvia przewalskii* Maxim herb [15]. Generally, phenolic acids show potent antioxidant activities and potential effects in protecting against brain and heart damage caused by ischemia reperfusion [24]. The small-molecule phenolic acids are likely to have higher activity and be more easily absorbed than other herbal ingredients. However, the concentration of these rare phenolic acids including przewalskinic acid A is very low in natural *S. miltiorrhiza* and related plants. Thus, the preparation of przewalskinic acid A from salvianolic acid B, which is a high-content water-soluble ingredient in *S. miltiorrhiza*, would be very important and useful for the research and development of the highly effective *S. miltiorrhiza* medicine and other herbal medicines.

To obtain more active and easily absorbed herbal ingredients, the biotransformations of natural products in herbs have been studied in our laboratory [4, 18, 20, 21].

Salvianolic acid B comprises two molecules of danshensu and two molecules of caffeic acid. During the enzyme reaction, salvianolic acid B was hydrolyzed into danshensu and an oligosalvianolic acid, i.e., przewalskinic acid A, which comprises two molecules of caffeic acid. However, dashensu is very common herbal ingredient [8, 13, 20, 21]. Danshensu can obtained by chemical methods, e.g., acid or alkali hydrolysis on danshen herb or salvianolic acid B [2, 3, 23], or by chemical synthesis [26]. However, these methods can not be used to prepare przewalskinic acid A, and very little research has been done on the preparation of przewalskinic acid A. In this paper, the preparation of przewalskinic acid A from salvianolic acid B was achieved using the crude enzyme from *Aspergillus oryzae* D30s.

Materials and methods

Materials

Reference substances of salvianolic acid B and danshensu were obtained from Dalian Bio-Chem Co. Ltd., Dalian, P.R. China. Dried roots of *S. miltiorrhiza* were obtained from Tianjin Tasly Institute, Tianjin, P.R. China. Plates of silica gel (60-F_{254}) were purchased from Merck, Germany. Sepabeads SP207 macroporous adsorption resin was purchased from Mitsubishi Chemicals Industries Co. Ltd., Japan. Pyridine-d_5 was purchased from Cortec Net, France.

Microorganism

The *A. oryzae* D30s strain obtained from the Culture Collection of Biotechnology Engineering of Dalian Polytechnic University (Dalian, Liaoning province, P.R. China) was isolated from Chinese traditional koji (Daqu in Chinese). The solid medium used for maintaining the strain was malt extract agar [5 % (w/v) Bacto-malt extract (Difco), 2 % (w/v) agar, tap water]. The medium used for enzyme production was tap water containing 1 % (v/v) extract of *S. miltiorrhiza* root and 5 % (w/v) Bacto-malt extract (Difco).

Preparation of salvianolic acid B from *S. miltiorrhiza* root

The preparation method of salvianolic acid B from *S. miltiorrhiza* root was based on an existing method [9], but a few changes were made. Dried root of *S. miltiorrhiza* (1 kg) was powdered and extracted three times with 5,000 ml of methanol for 24 h at room temperature. The extract was filtered under vacuum through filter paper. The filtrates were combined and concentrated by vacuum evaporation at 38 °C [9]. Then the crude extract containing salvianolic acid B was removed the ester using ether and petroleum ether, and was dissolved in water (3,000 ml) and subjected to chromatography on a column of Sepabeads SP207 macroporous adsorption resin (1,500 ml, 4 ml/min, 3 times), eluted with water (6 times the column volume) to remove impurities, then eluted with 60 % ethanol (4 times the column volume). The eluants were combined and concentrated by vacuum evaporation to obtain salvianolic acid B.

Preparation of enzyme inducer (extract of *S. miltiorrhiza* root)

The *S. miltiorrhiza* extract was used as an inducer for the enzyme production, and it was prepared by water extraction as follows: the 100 g dried root of *S. miltiorrhiza* was crushed and added to 600 ml of tap water, boiled for about 7 h, and filtered to remove precipitate, and adjusted to a final volume of 300 ml.

Preparation of crude enzyme

Two loopfuls of *A. oryzae* D30s strain was inoculated into 200 ml of liquid medium, and cultured for 96 h at 30 °C with shaking at 140 rpm in 5 % (w/v) malt extract medium containing 1 % (v/v) extract of *S. miltiorrhiza* root as the enzyme inducer. To obtain the crude enzyme, the culture was centrifugated to remove insoluble substances. Then, the supernatant enzyme was precipitated with $(NH_4)_2SO_4$ (75 % saturation) and stored at 4 °C for 12 h, and precipitated enzyme protein was collected by centrifugation, dissolved in 0.02 M acetate buffer (pH 5.0, 1/10 volume of the

initial culture), and dialyzed in the same buffer for 24 h. The dialyzed enzyme solution was centrifugated to remove the insoluble impurities and diluted with the same buffer to half the volume of the initial culture. The prepared crude enzyme solution was stored at 4 °C for subsequent experiments.

Assays of enzyme activity

For assaying the enzymatic activity, 1 ml crude enzyme was mixed with the same volume of 16 mg/ml (about 22 mM or 1.6 %) salvianolic acid B (substrate) in 0.02 M acetate buffer (pH 5.0), and reacted at 40 °C for 14 h. Thereafter, 2 ml of water-saturated *n*-butanol was added to the reaction mixture to stop the enzyme reaction. The reaction product in the *n*-butanol layer was analyzed by TLC and HPLC. The spots on the silica plate were scanned using a Shimadzu CS-930 spectrophotometer (Shimadzu Corp., Kyoto, Japan). One unit of enzyme activity was defined as the amount of enzyme that produced 1 mmol of product II (przewalskinic acid A) per hour under optimal conditions.

Preparation przewalskinic acid A

To obtain large amounts of przewalskinic acid A, the enzyme reaction was carried out in a 5-l reactor (RAT-1-5D, Shanghai shensun Biological Technology Co., Ltd., Shanghai, P.R. China). Thus, 2 l of crude enzyme was mixed with the same volume of salvianolic acid B (16 mg/ml) to 8 mg/ml (about 11 mM) of final substrate concentration, and reacted under the above enzyme reaction conditions. After the enzyme reaction was stopped by the addition of alcohol to 75 % concentration, and then insoluble material by was removed by centrifugation. The supernatant was concentrated by vacuum evaporation to obtain reaction products containing przewalskinic acid A and danshensu. The reaction products were separated and purified by chromatography on a column of Sepabeads SP207 macroporous adsorption resin (volume, 30 times the substrate weight); the enzyme reaction products were dissolved in distilled water to 5 % concentration, and were absorbed on a column of SP207 macroporous adsorption resin, and eluted with water (six times the column volume) to remove impurities; then eluted with 30 % ethanol (6–8 times the column volume) to obtain the danshensu solution, and eluted with 50 % ethanol (six times the column volume) to get the przewalskinic acid A solution. Then, the przewalskinic acid A and danshensu solutions were concentrated by vacuum evaporation to get pure przewalskinic acid A and pure danshensu, respectively.

TLC analysis

TLC analysis was performed on silica gel plates developed with ethyl acetate/formic acid (10:1, v/v). Visualization of the TLC plates was performed by spraying with 5 % $FeCl_3$ reagent, followed by heating at 110 °C for 10 min.

HPLC analysis

HPLC analysis was performed on a Waters 2695 HPLC instrument. A Kromasil C18 column (Φ4.6 × 250 mm, 5 μm, Dalian Zhonghuida Scientific Instrument Corp., P.R. China) was used to analyze the enzymatic reaction product, and a mobile phase of methanol (solvent A)/distilled water containing 1.0 % acetic acid (solvent B) at flow rate of 1.0 ml/min was used as follows: 0–5 min, A was 5 %; 5–15 min, A from 5 to 15 %; 15–30 min, A from 15 to 50 %; 30–50 min, A from 50 to 90 %; 50–60 min, A from 90 to 100 %; monitoring at 286 nm. The injection volume was 10 μl and the column temperature was set at 35 °C.

UPLC–MS analysis

Enzymolysis products from salvianolic acid B were examined by ultra-performance liquid chromatography–mass spectrometry (UPLC–MS) with a UPLC-Q/Tof Premier™ Micromass instrument (Waters Corp., Milford, USA). An Acquity UPLC HSS T3 chromatography column (Φ2.1 × 100 mm, 1.8 μm, Waters Corp.) was used for UPLC with a gradient elution system which consisted of acetonitrile (solvent A) and distilled water containing 0.2 % methanoic acid (solvent B). The measurements of mass were further carried out on a Q-Tof Premier mass spectrometer between 100 and 1,500 Da under negative-ionization conditions. A spray voltage of 4.5 kV was employed and the temperature of heated transfer capillary was set at 350 °C.

NMR analysis

The structure of enzymolysis product II (przewalskinic acid A) from salvianolic acid B was analyzed using NMR. The product was dissolved in pyridine-d_5, and the NMR spectra were recorded by using a Bruker Avance 600 (^{1}H, 600 MHz; ^{13}C, 150 MHz) NMR spectrometer (Switzerland).

Results and discussion

For preparation of salvianolic acid B from *S. miltiorrhiza*, 149 g of crude salvianolic acid B was extracted with methanol from 1 kg dried root of *S. miltiorrhiza*. Separation by chromatography over Sepabeads SP207 afforded about 30.3 g of purified salvianolic acid B. The extraction experiment was repeated four times; the average extraction yield of salvianolic acid B was 3.1 %, the purity was 91.2 %, and

the produced salvianolic acid B was used as a substrate in the subsequent biotransformation.

The effect of inducer concentration on enzyme production of *A. oryzae* D30s was studied by adding extracts of *S. miltiorrhiza* with different concentrations [0.2, 0.4, 0.6, 0.8, 1.0, 1.2, 1.4, 1.6, and 1.8 % (v/v)]. The enzyme production increased at the inducer concentration varied from 0.2 to 1.0 %, and did not increase at 1.0–1.8 %; so, an inducer concentration of 1 % extract of *S. miltiorrhiza* root was chosen. Good enzyme production was obtained from the culture of *A. oryzae* D30s after culture at 40 °C for 96 h in the medium containing 5 % (w/v) malt extract and 1 % (v/v) extract of *S. miltiorrhiza* extract as inducer. The catalytic reaction of salvianolic acid B by the crude enzyme was carried out for 14 h at 40 °C. The products were then analyzed by TLC and HPLC (Fig. 1). As shown in Fig. 1a, the substrate (salvianolic acid B) was transformed by the crude enzyme into two products, which were considered to be danshensu and przewalskinic acid A (Fig. 1a, line 5). As shown from the HPLC traces in Fig. 1b, c, salvianolic acid B was converted into two products: danshensu (product I, peak 1) and product II. Because of the lack of reference standard, product II (peak 2) was further identified by UPLC–MS and NMR.

Enzyme reaction product II was separated from the enzyme reaction mixtures by using a column of Sepabeads SP207 and purified by crystallization. The UPLC–MS and NMR data of the enzyme reaction product II are as follows: ESI–MS (negative): *m/z* 313.04 $[M-COOH]^-$, i.e., the molecular mass of the product was 358; ^{1}H NMR (600 MHz, pyridine-d_5): δ_H 7.88 (1H, *d*, J = 15.6 Hz, 7-CH), 7.21 (1H, *d*, J = 8.4 Hz, 5′-CH), 6.90 (1H, *d*, J = 1.2 Hz, 2′-CH), 6.80–6.86 (3H, *m*, 5-CH, 8-CH, 6′-CH), 6.36 (1H, *d*, J = 15.6 Hz, 6-CH), 5.96 (1H, *d*, J = 5.4 Hz, 7′-CH), 4.32 (1H, *d*, J = 5.4 Hz, 8′-CH), 3.88 (5H, br, –OH); for ^{13}C NMR spectral data, see Table 1. These NMR data corresponded with those of a previous report [15] and authentic przewalskinic acid A (Fig. 2).

Salvianolic acid B comprises two molecules of danshensu and two molecules of caffeic acid. During the enzyme reaction, salvianolic acid B was hydrolyzed into danshensu and an oligosalvianolic acid, i.e., przewalskinic acid A, which consists of two molecules of caffeic acid. Therefore, salvianolic acid B was hydrolyzed by the enzyme from *A. oryzae* D30s to danshensu and przewalskinic acid A as shown in Fig. 2. Thus, it is possible to produce the rare przewalskinic acid A from salvianolic acid B of *S. miltiorrhiza* using the crude enzyme from the *A. oryzae* strain.

In the preparation of przewalskinic acid A, the enzyme reaction was performed in a 5-l reactor. The 2 l of crude enzyme was mixed with the same volume of 16 mg/ml salvianolic acid B (final substrate concentration of 8 mg/ml

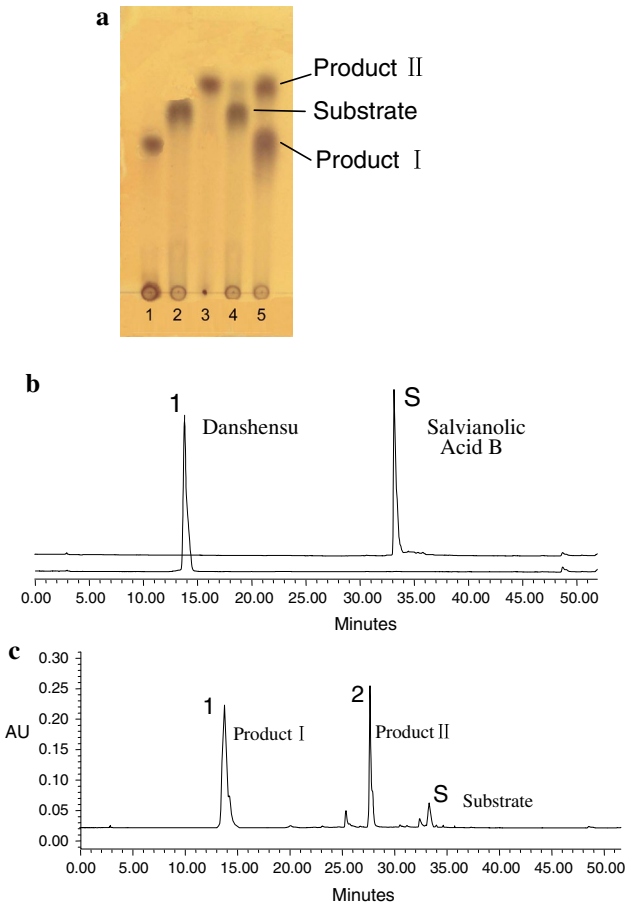

Fig. 1 The enzymolysis products from salvianolic acid B by TLC and HPLC. **a** TLC analysis of the enzymolysis products from salvianolic acid B: *1* standard danshensu, *2* standard salvianolic acid B, *3* product II after separation, *4* substrate salvianolic acid B, *5* the enzymolysis products from salvianolic acid B. Developing solvent, ethyl acetate/formic acid (10:1, v/v); chromogenic agent, 5 % $FeCl_3$. HPLC analyses of **b** standards: *1* standard of danshensu, *S* standard of salvianolic acid B; **c** enzymolysis products from salvianolic acid B: *1* product I (danshensu), *2* product II (przewalskinic acid A), *S* substrate salvianolic acid B. Enzyme, 10 U/ml; substrate, 8 mg/ml; reacted at 40 °C and pH 5.0 for 12 h

in the reaction mixture) in the batch reactor. After being reacted for 14 h, salvianolic acid B was almost completely transformed to danshensu and przewalskinic acid A. The alcohol was added to 75 % concentration to stop the enzyme reaction, and then insoluble material was removed by centrifugation. The supernatant was concentrated by vacuum evaporation. The reaction products were separated by the macroporous adsorption resin column (Φ10 × 30 cm); the enzyme reaction products were dissolved in distilled water to 3–5 % concentration, absorbed on a column (Φ10 × 30 cm) of SP207 macroporous adsorption resin, and eluted with water (6 times the column volume) to remove impurities, then eluted with 30 % ethanol (6–8 times the column volume) to obtain danshensu, and eluted with 50 %

Table 1 Comparison of ^{13}C NMR spectroscopic data of enzyme reaction product, przewalskinic acid A, with literature data (δ in ppm)

Carbon site	δ (this work)	δ [reference 15]	Carbon site	δ (this work)	δ [reference 15]
C-1	129.6	127.4	C-1′	125.3, C	124.8
C-2	134.8	133.8	C-2′	114.0, CH	113.4
C-3	149.1	148.8	C-3′	146.8, C	146.6
C-4	146.8	146.8	C-4′	144.8, C	144.9
C-5	116.6	116.4	C-5′	121.4, CH	121.5
C-6	118.7	118.2	C-6′	118.7, CH	118.2
C-7	143.3	143.4	C-7′	90.2, CH	88.9
C-8	118.0	117.6	C-8′	60.4, CH	57.7
C-9	172.4	170.7	C-9′	178.6, C	175.1

In this work, assignments were based on ^{1} H, ^{13}C, DEPT, COSY, HSQC, and HMBC NMR experiments. Ref. [15], spectra were obtained at 67.8 MHz in pyridine-d_5

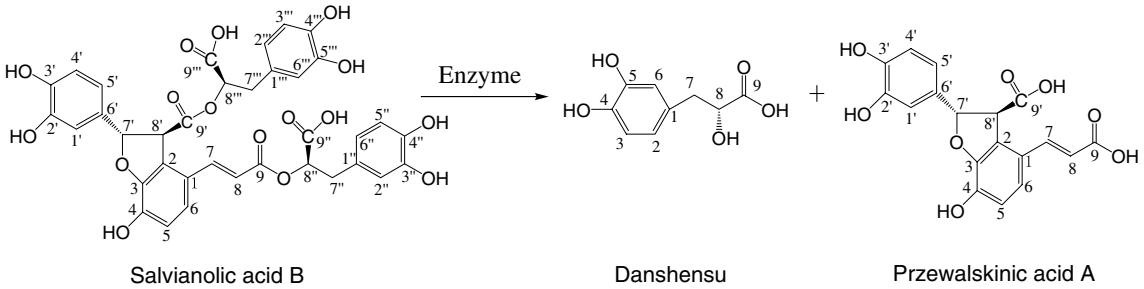

Fig. 2 Biotransformation of salvianolic acid B by the enzyme from *A. oryzae* D30s

ethanol (6 times the column volume) to get przewalskinic acid A. Then, the przewalskinic acid A and danshensu solutions were concentrated by vacuum evaporation and precipitated to get pure przewalskinic acid A and pure danshensu, respectively. This experiment was carried out three times. The products of 31.3 g przewalskinic acid A (91.0 % purity) and 13.1 g danshensu (95 % purity) were obtained from 75 g of salvianolic acid B. The yield of przewalskinic acid A was 86 % of the theoretical yield, but the yield of danshensu was only 33 % of the theoretical yield. The latter might be because of the instability of danshensu in the enzyme reaction (pH 5.0), and further studies on the effect of pH on the yield of danshensu are needed.

Conclusion

The rare przewalskinic acid A was prepared by biotransformation for the first time. The crude enzyme from *Aspergillus oryzae* D30s can transform salvianolic acid B to danshensu and przewalskinic acid A. The yield of przewalskinic acid A from salvianolic acid B was 86 % of the theoretical yield, proving that the przewalskinic acid A production was very successful. This approach may be applicable for the practical preparation of przewalskinic acid A for medicinal purposes. However, the yield of danshensu was only 33 % of the theoretical yield and the method requires further studies.

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