J Ind Microbiol Biotechnol (2013) 40:427–436
DOI 10.1007/s10295-013-1246-x

BIOCATALYSIS

Preparation of progenin III from total steroidal saponins of *Dioscorea nipponica* Makino using a crude enzyme from *Aspergillus oryzae* strain

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Received: 9 November 2012 / Accepted: 18 February 2013 / Published online: 8 March 2013

Abstract Progenin III, one of the most active spirostanol saponins, is a potential candidate for anti-cancer therapy due to its strong antitumor activity and low hemolytic activity. However, the concentration of progenin III is extremely low in natural *Dioscorea* plants. In this paper, the progenin III production from total steroidal saponins of *Dioscorea nipponica* Makino was studied using the crude enzyme from *Aspergillus oryzae* DLFCC-38. The crude enzyme converting total steroidal saponins into progenin III was obtained from the *A. oryzae* DLFCC-38 culture. For enzyme production, the strain was cultured for 72 h at 30 °C with shaking at 150 rpm in 5 % (w/v) malt extract medium containing 2 % (v/v) extract of *D. nipponica* as the enzyme inducer. The crude enzyme converted total steroidal saponins into major progenin III with a high yield when the reaction was carried out for 9 h at 50 °C and pH 5.0 with the 20 mg/ml of substrate. In the preparation of progenin III, 117 g of crude progenin III was obtained from 160 g of substrate, and the crude product was purified with silica gel column to obtain 60.3 g progenin III of 93.4 % purity.

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Keywords *Dioscorea nipponica* Makino · Progenin III · Enzymatic preparation · *Aspergillus oryzae* · Steroidal saponins

Introduction

Steroidal saponin is one of the most important ingredients, or secondary metabolites in many plants, such as *Dioscorea*, *Paris*, and *Trillium* genus plants [2, 14, 23, 38, 42], and it is divided into two major groups, furostanosides and spirostanosides. These steroidal saponins were reported to possess a variety of pharmacological activities such as anti-platelet aggregation, anti-tumor, anti-diabetic, anti-hyperlipidemic, and anti-oxidative [28, 32–35]. Among them, cytotoxicity against tumor cells has received more and more attention, but the structure–activity relationship studies on steroidal saponins indicated that the cytotoxicity is highly sensitive to the change of their structures [22, 29, 36]. Most of the furostanosides bearing an additional monosaccharide at C-26 (compared to spirostanoside) only showed some anti-cancer activity [8, 22], whereas spirostanosides such as dioscin, progenin III (prosapogenin A of dioscin, Paris V) and progenin II (prosapogenin B of dioscin) with low sugar chain exhibited strong cytotoxic activity [1, 3]. Among these three spirostanosides, progenin III exerts the strongest cytotoxicity against several human cancer cells [24], and it showed low hemolytic activity, which might be considered as a lead compound for potent antitumor agents [36]. In addition, progenin III showed strong antimicrobial activity [18, 30, 31]. Progenin III has been found in *Dioscorea*, *Paris*, and *Trillium* genus plants [3, 18, 19, 23, 25, 30, 31, 39, 40, 42], but the content of progenin III is very low, and the content of progenin III in the *Paris* and *Trillium* genus plants was lower than 0.53 mg/g

[13, 23, 42]. Thus, the preparation of progenin III from high-content steroidal saponins in plants would be very important for pharmological study and drug development.

The root of *Dioscorea nipponica* Makino, called Chuanshanlong in Chinese, is an important medicinal herb that is widely used in traditional Chinese medicine for its antibechic, antiasthmatic, antirheumatic, demulcent, and antilipemic properties [21]. The dried root of *D. nipponica* Makino contains major steroidal saponins with long sugar chains such as protodioscin and dioscin [12, 14, 16, 17] and these steroidal saponins could be converted into corresponding spirostanosides with low sugar chains by microbial transformation [27]. Thus, the total steroidal saponins in *D. nipponica* Makino may be a useful material for preparation of rare steroidal saponins with low sugar chains. Previous studies of modification of natural steroids from the *Dioscorea* genus focused on preparation of diosgenin [6, 9, 15, 20, 43] or partial modification by microbial biotransformation [5, 26, 41], but until now, there has been no study on the enzymatic preparation of progenin III by using crude microbial enzymes.

In this paper, the preparation of the rare progenin III from the total steroidal saponins of *D. nipponica* Makino was achieved using enzymes from *Aspergillus oryzae* DLFCC-38. To obtain higher production of the rare progenin III, the enzyme production from *A. oryzae* DLFCC-38 and reaction conditions for the progenin III preparation were studied.

Materials and methods

Materials

Reference substances of protodioscin, dioscin, gracillin, progenin III, and diosgenin were prepared in our laboratory [6, 26] or obtained from Shangyihua-Huagong Co., Ltd, Shanghai, P.R. China. Dried roots of *Dioscorea nipponica* Makino were purchased from a local commercial pharmacy whose origin is Liaoning province, P.R. China. Plates of silica gel (60-F_{254}) were obtained from Merck, Germany. AB-8 macroporous adsorption resin (polystyrene resin) and D-280 strong basic anion exchanger resin (styrene-DVB resin) were purchased from the Chemical Plant of Nankai University, Tianjin, P.R. China. The silica gel of 300–400 mesh was purchased from Qingdao Haiyang Chemical Co., Ltd, Shandong province, P.R. China.

Microorganism

The fungal strain of *Aspergillus oryzae* DLFCC-38 [10] 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 strain was identified to be the same strain as *Aspergillus oryzae* AS 3.800 (Culture Collection, Institute of Microbiology, Chinese Academy of Sciences, AS, Beijing, China). 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 5 % (w/v) Bacto-malt extract (Difco) and 2 % (v/v) extract of *D nipponica* Makino.

Preparation of total steroidal saponins

The dried root of *Dioscorea nipponica* Makino (slice of 2-mm thickness) was extracted three times with 80 % ethanol at room temperature for 24 h. The resulting ethanol extract was filtered through filter-paper, collected, and concentrated under reduced pressure in a rotary evaporator to remove ethanol. The concentrated raffinate was degreased three times with 30 % (v/v) petroleum ether (bp 60–90 °C), and then applied on the AB-8 macroporous adsorption resin column to absorb the saponins. The column was firstly washed with six-column volumes of water to remove soluble impurities such as sugars, and then the saponins adsorbed on the resin was eluted with five-column volumes of 85 % ethanol. The ethanol eluate was further decolorized with the column of D-280 strong basic anion exchanger resin, and the decolorized solution was dried by vacuum distillation to obtain the total steroidal saponins.

Preparation of the enzyme inducer (extract of *D. nipponica* Makino)

The *D. nipponica* Makino extract was used as an inducer for enzyme production. The *D. nipponica* Makino extract was prepared by the method of water extraction, the 100 g sliced root of *D. nipponica* Makino was added into 600 ml of tap water, boiled for about 7 h, and filtered to remove precipitate, and adjusted to a final volume of 300 ml.

Optimization of enzyme production

For optimization of enzyme production, all optimization experiments were carried out by incubating two loopfuls of *A. oryzae* DLFCC-38 with 200 ml liquid medium in 1,000-ml flasks.

The effect of shaking speed on enzyme production was determined at different shaking speeds such as 100, 120, 150, and 200 rpm, and the cultivation was carried out at 30 °C for 72 h in 5 % (w/v) malt extract medium containing 2 % (v/v) extract of *D. nipponica* Makino as inducer.

The effect of temperature on enzyme production was investigated at different temperatures (25, 30, and 37 °C), and the strain was cultured in 5 % (w/v) malt extract medium containing 2 % (v/v) extract of *D. nipponica* Makino extract for 72 h at 150 rpm.

The effect of malt concentration on enzyme production was determined by incubating the strain in medium with different concentrations of malt extract [1, 3, 5, 7, and 9 % (w/v)], and 2 % (v/v) of *D. nipponica* Makino extract as inducer at 30 °C, 150 rpm for 72 h.

The effect of inducer concentration on enzyme production was investigated by incubating the strain in 5 % (w/v) malt extract medium containing various concentrations of *D. nipponica* Makino extract [0.0, 0.5, 1.0, 1.5, 2.5, 3.0, 3.5, 4.0 % (v/v)] at 30 °C, 150 rpm for 72 h.

The culture time effect on enzyme production was examined by cultivation for 12, 24, 36, 48, 60, 72, 84, 96, 108, and 120 h as described before [6], and the cultivation was carried out at 30 °C, 150 rpm in the medium containing 5 % (w/v) malt extract and 2 % (v/v) extract of *D. nipponica* Makino. Three independent experiments, with three replicates, were carried out for each cultivation treatment.

Preparation of crude enzyme

The preinoculum was prepared by incubating two loopfuls of *A. oryzae* DLFCC-38 with 200 ml of liquid malt extract (5 % w/v) medium for 24–48 h under the optimum culture conditions. To produce enzymes, 4 ml of this preinoculum was added to a 1,000-ml flask with 200 ml liquid medium containing 5 % (w/v) malt extract and 2 % (v/v) extract of *D. nipponica* Makino as enzyme inducer, and fermented under the optimum culture conditions.

The culture was centrifugated to remove cells and other non-dissolving impurities. The supernatant enzyme was precipitated with ammonium sulphate (75 % saturation) and stored overnight at 4 °C. The precipitate enzyme protein was collected by centrifugation, dissolved in 0.02 M, pH 5.0 acetate buffer (one-tenth volume of initial culture), and dialyzed against the same buffer. The dialyzed enzyme solution was centrifugated to remove the non-dissolving impurities and diluted with the same buffer to half volume of the initial culture of *A. oryzae* DLFCC-38. A total of 8 l culture of *A. oryzae* DLFCC-38 was obtained from the cultured 40 flasks simultaneously, and the culture of 8 l was concentrated twofold to obtain 4 l crude enzyme solution. Then enzyme solution was stored at 4 °C and used as crude enzyme for following experiments.

Assays of enzyme activity

One milliliter of crude enzyme was mixed with the same volume of 40 mg/ml total steroidal saponins in 0.02 M, pH 5.0 acetate buffer, and reacted at 50 °C for 24 h with a shaking speed of 120 rpm. Then, 2 ml of *n*-butanol saturated by water was added to the reaction mixture to stop enzyme reaction. The reaction product in the *n*-butanol layer was analyzed by the methods of thin-layer chromatography (TLC) and high-performance liquid chromatography (HPLC). One unit of enzyme activity was defined as the amount of enzyme that produced 1 μmol of progenin III per hour at optimal condition.

Optimization of enzymatic reaction conditions

The effect of pH on the enzyme reaction was studied by carrying out the enzyme reaction for 9 h at 50 °C with different buffers at 0.02 M (pH 2.0, 3.0, 4.0, 5.0, 6.0, 7.0, 8.0, 9.0, and 10). For studying the effect of temperature, the enzyme reaction was carried out at different temperatures (20, 30, 40, 50, 60, and 70 °C) for 9 h under optimal pH. To study the effect of substrate concentration and reaction time, the crude enzyme was reacted with total steroidal saponins at final concentrations of 5, 10, 15, 20, 25, and 30 mg/ml for various time periods (1, 3, 5, 7, 9, 11 and 13 h) under the optimum reaction conditions, respectively. Moreover, the effect of metal ions on enzyme reaction was investigated using metal ions such as K^+, Na^+, Ca^{2+}, Mg^{2+}, Zn^{2+}, Cu^{2+}, and Fe^{3+}. The crude enzyme solutions containing each individual metal ions was reacted with substrate total steroidal saponins (final concentration, 20 mg/ml) under the optimal pH and temperature for 9 h.

In the above experiments, *n*-butanol saturated by water was added to the reaction mixture to stop enzyme reaction, and the reaction production in the *n*-butanol layer was analyzed by the methods of TLC and HPLC. Three independent experiments, with three replicates, were carried out for each reaction. The yield of progenin III by enzyme reaction was calculated as follows:

$$\text{yield } (\%) = \frac{\text{progenin III produced (mg)}}{\text{total steroidal saponins added (mg)}} \times 100$$

Scale-up preparation of progenin III by enzyme reaction

To obtain the progenin III, the batch-reaction was performed twice in the 5-L reactor (RAT-1-5D, Shanghai shensun Biological Technology Co., Ltd., Shanghai, P.R. China). For each reaction, the 2 l of crude enzyme was mixed with the same volume of 40 mg/ml total steroidal saponins in 0.02 M acetate buffer (pH 5.0), and reacted under the optimum conditions.

After enzyme reaction, the progenin III products were precipitate. So the precipitate of progenin III was collected, washed by water, and dried under vacuum to obtain the crude progenin III. The crude product was further separated

by silica gel column chromatography, and the ratio of crude progenin III to the silica gel (300–400 mesh, Qingdao Haiyang Chemical Co., Ltd, P.R. China) was 1: 20. The elution was performed with gradient $CHCl_3$: CH_3OH: H_2O (9.5: 0.5: 0.1, 9: 1: 0.1, 8.5: 1.5: 0.5, and 8: 2: 0.5, lower phase).

TLC analysis

The TLC was performed on Merck silica-gel plates developed by $CHCl_3$/MeOH/H_2O (70:30:5, by vol., lower phase). Visualization of the TLC plates was performed by 10 % H_2SO_4 spray reagent, followed by heating at 110 °C for 10 min. The spots of produced saponins on the TLC were scanned using a Shimadzu CS-930 spectrophotometer [6].

HPLC analysis

HPLC analysis was performed on a Waters 2695 HPLC instrument using a Kromasil C18 column (5 μm, 200 × 4.6 mm, Dalian Zhonghuida Scientific Instrument, P.R. China), and CH_3CN (A)-H_2O (B) as mobile phase at flow rate of 1.0 ml/min was used as follows, 0–5 min, A from 20 to 30 %, 5–20 min, A from 30 to 40 %; 20–25 min, A from 40 to 46 %; 25–50 min, A 46 %; 50–60 min, A from 46 to 70 %; 60–70 min, A from 70 to 90 %; 70–90 min, A 90 %; monitoring at 206 nm.

NMR analysis

The product, progenin III, structure was analyzed using NMR. The NMR spectra for the main product, progenin III,

Table 1 Comparison of ^{13}C NMR spectroscopic data of progenin III with literature data (δ in ppm)

Carbon site	This work	Ref. [11]	Carbon site	This work	Ref. [11]	Ref. [37]
Aglycone						
Moiety			3-*O*-Glc			
C-1	37.6	37.6	C-1′	102.6	100.5	100.6
C-2	30.3	30.3	C-2′	78.4	78.0	79.6
C-3	78.4	78.2	C-3′	76.8	79.7	78.0
C-4	39.5	39.1	C-4′	75.6	72.0	72.1
C-5	141.0	141.0	C-5′	77.3	78.2	78.0
C-6	121.9	121.8	C-6′	61.7	62.8	62.9
C-7	32.4	32.4	Rha (1→2)			
C-8	31.8	31.8	C-1″	102.8	102.0	101.9
C-9	50.4	50.5	C-2″	72.8	72.6	72.5
C-10	37.2	37.2	C-3″	73.0	72.9	72.9
C-11	21.3	21.2	C-4″	74.1	74.3	74.2
C-12	40.0	40.0	C-5″	70.5	69.5	69.4
C-13	40.6	40.6	C-6″	18.7	18.7	18.6
C-14	56.8	56.8				
C-15	32.3	32.3				
C-16	81.2	81.2				
C-17	63.0	63.0				
C-18	16.5	16.4				
C-19	19.6	19.5				
C-20	42.1	42.1				
C-21	15.2	15.0				
C-22	109.4	109.3				
C-23	32.0	31.9				
C-24	29.4	29.3				
C-25	30.8	30.7				
C-26	67.0	67.0				
C-27	17.5	17.3				

In this work, assignments were based on ^{1}H, ^{13}C, DEPT, COSY, HSQC, and HMBC NMR experiments, and ^{13}C spectra were obtained at 150 MHz in pyridine-d_5. Ref. [11], spectra were obtained at 125 MHz in pyridine-d_5. Ref. [37], spectra were obtained at 25 MHz in pyridine-d_5. *Glc* glucose, *Rha* rhamnose

were recorded in pyridine-d_5 on Bruker AVANCE 600 (^{1}H: 600 MHz; ^{13}C: 150 MHz) spectrometer. White powder; ^{1}H-NMR (600 MHz, pyridine-d_5): δ_H 0.59 (3H, s, 27-CH_3), 0.71 (3H, s, 18-CH_3), 0.80 (3H, s, 19-CH_3), 1.02 (3H, s, 21-CH_3), 1.60 (3H, s, Rha-CH_3), 4.83 (1H, s, Glc H-1), 5.76 (1H, s, Rha H-1), 5.21 (H, br s, H-6); for ^{13}C NMR spectral data, see Table 1.

Results and discussion

Biotransformation of total steroidal saponins

The total steroidal saponins of 250 g (pale yellow powder) were obtained from 4 kg of dried roots of *D. nipponica* Makino, and the extraction yield was about 6.3 %. The total steroidal saponins were analyzed by the method of HPLC, and the result is shown in Fig. 1a. The total steroidal saponins consist of 44.2 % protodioscin, 8.5 % protogracillin, and 29.1 % dioscin. It is shown that the main saponins of *D. nipponica* Makino are protodioscin and dioscin, but the content of progenin III is very low (Fig. 1a). The total steroidal saponins from *D. nipponica* Makino was used as substrate in the following experiments.

The crude enzyme, obtained from *A. oryzae* DLFCC-38 culture after cultivation at 30 °C and 150 rpm for 72 h in the medium containing 5 % (w/v) malt extract and 2 % (v/v) extract of *D. nipponica* Makino extract as inducer, converted the total steroid saponins from *D. nipponica* Makino to produce progenin III after reacted for 24 h at 50 °C (Fig. 1).

It was shown from Fig. 1b (TLC) that the substrate total steroidal saponins (major protodioscin, dioscin, and a small amount of protogracillin) was transformed by the enzyme to major progenin III with a small amount of gracillin, dioscin, and aglycone after reaction for 24 h (Fig. 1b, line 8).

It was shown from the HPLC result (Fig. 1a) that the major saponins of substrate, i.e., protodioscin (peak 1), protogracillin (peak 2) and dioscin (peak 3) were converted into major progenin III (peak 5) containing a small amount of dioscin (peak 3), gracillin (peak 4) and aglycone (peak 6), after reaction for 24 h.

From the results in Fig. 1, there are some possible transformation pathways of the total steroidal saponins by the enzyme from *A. oryzae* DLFCC-38 as shown in Fig. 2. The main pathway I, the 26-*O*-β-D-glc of protodioscin was hydrolyzed into dioscin, and the 3-*O*-α-L-(1→4)-rha of dioscin was further hydrolyzed to progenin III. The possible pathway II, the 3-*O*-α-L-(1→4)-rha of protodioscin was firstly hydrolyzed into protobioside, and the 26-*O*-β-D-glc of protobioside was further hydrolyzed to progenin III.

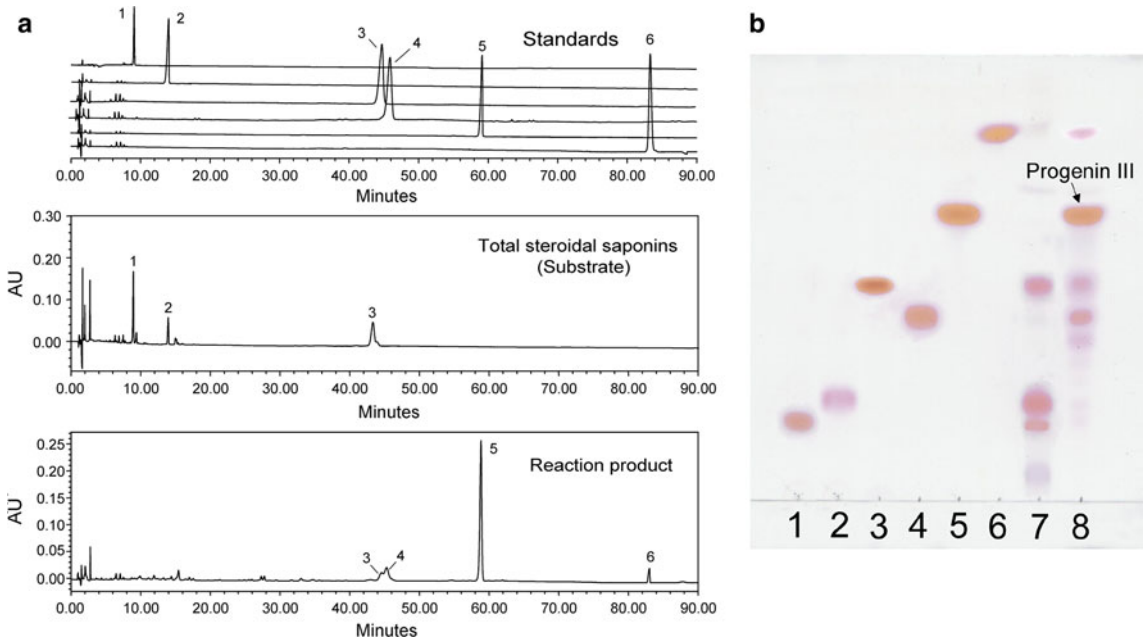

Fig. 1 Biotransformation of total steroidal saponins by crude enzyme from *A. oryzae* DLFCC-38. **a** HPLC analysis of the biotransformation product of total steroidal saponins by crude enzyme from *A. oryzae* DLFCC-38. *1–6*, standard steroidal saponins: protodioscin, protogracillin, dioscin, gracillin, progenin III, diosgenin. **b** TLC analysis of the biotransformation product of total steroidal saponins by crude enzyme from *A. oryzae* DLFCC-38. *1–6*, standard steroidal saponins: protogracillin, protodioscin, dioscin, gracillin, *progenin* III, diosgenin; *7*, substrate total steroidal saponins; *8*, reaction product. The crude enzyme from *A. oryzae* DLFCC-38 was reacted with total steroidal saponins (final concentration, 20 mg/ml) at 50 °C for 24 h. Developing solvent for TLC analysis: $CHCl_3$/MeOH/H_2O (70:30:5, by vol., lower phase)

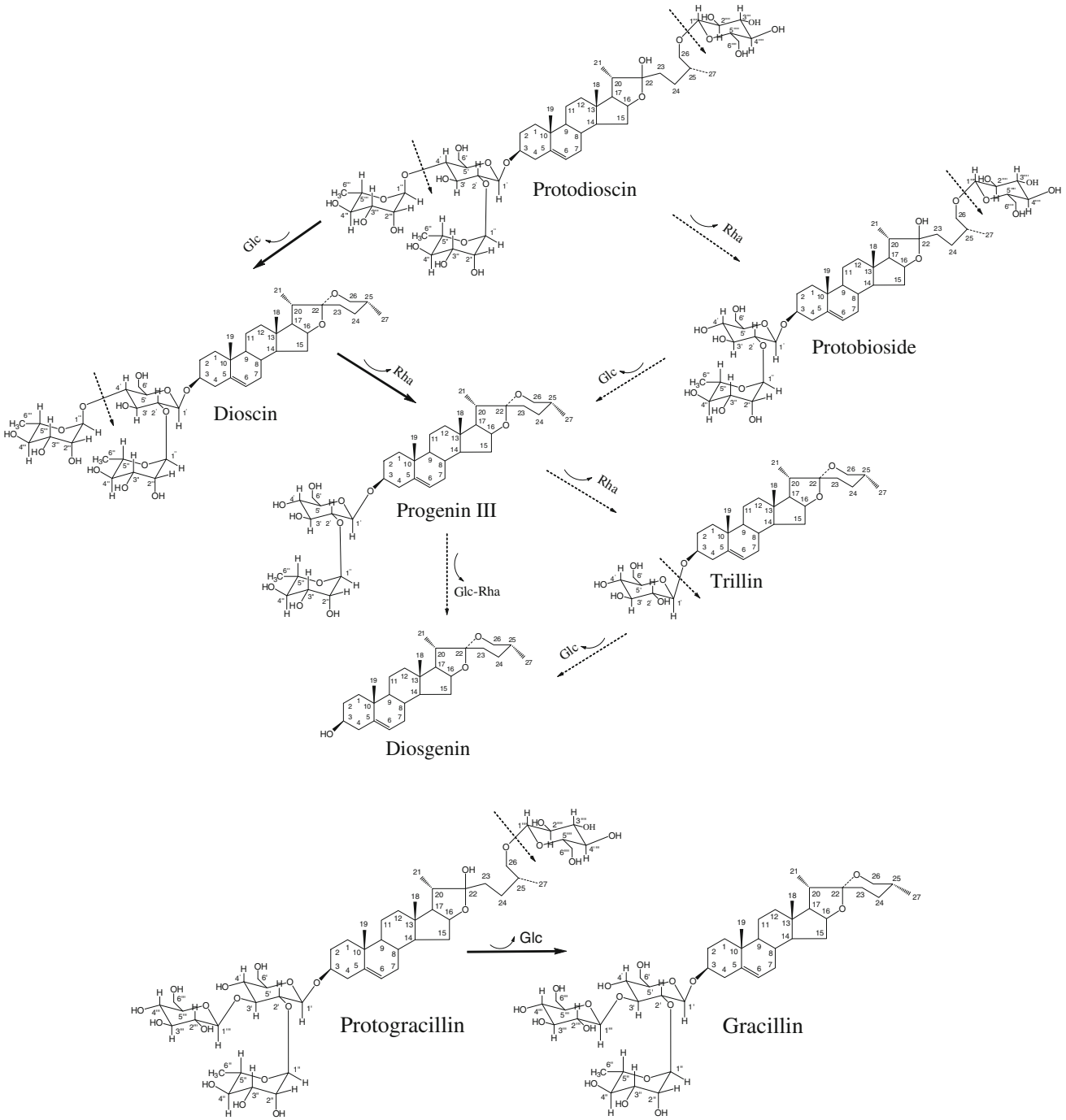

Fig. 2 Proposed biotransformation pathway of the main saponins in total steroidal saponins by crude enzyme from *A. oryzae* DLFCC-38. The *solid arrow* (→) marks the main reaction pathway

Thereafter, the 3-*O*-α-L-rha-(1→4)-β-D-glc of progenin III was slightly hydrolyzed into aglycone (diosgenin) after more than 24 h. Moreover, the 26-*O*-β-D-glc of protogracillin was hydrolyzed to gracillin as shown in Fig. 2. The scientific names of these steroidal saponins are shown in Table 2.

These properties of the crude enzyme from *A. oryzae* DLFCC-38 are different from the previously reported results of *A. oryzae* AS 3.951 (Culture Collection, Institute of Microbiology, Chinese Academy of Sciences, AS, Beijing, China) [27]. In the literature [27], the protodioscin was directly added into *A. oryzae* AS 3.951 culture (soybean sprout medium), and the 26-*O*-β-D-glc and 3-*O*-α-L-(1→2)-rha of protodioscin were hydrolyzed into progenin II. However, the enzyme from *A. oryzae* DLFCC-38 hydrolyzed protodioscin of total steroidal saponins into

Table 2 Scientific names of steroidal saponins

Name	Scientific name
Diosgenin	3β-hydroxy-5-spirostene
Protodioscin	26-*O*-β-D-glucopyranosyl-(25*R*)-22-hydroxyl-5-ene-furostan-3β,26-diol-3-*O*-α-L- rhamnopyranosyl-(1→2)-[α-L-rhamnopyranosyl-(1→4)]-β-D-glucopyranoside
Protobioside	26-*O*-β-D-glucopyranosyl-(25*R*)-22-hydroxyl-5-ene-furostan-3β,26-diol-3-*O*-α-L- rhamnopyranosyl-(1→2)-β-D-glucopyranoside
Dioscin	Diosgenin-3-*O*-α-L-rhamnopyranosyl-(1→2)-[α-L-rhamnopyranosyl-(1→4)]- β-D-glucopyranoside
Progenin III	Diosgenin-3-*O*-α-L-rhamnopyranosyl-(1→2)-β-D-glucopyranoside
Progenin II	Diosgenin-3-*O*-α-L-rhamnopyranosyl-(1→4)-β-D-glucopyranoside
Trillin	Diosgenin-3-*O*-β-D-glucopyranoside
Protogracillin	26-*O*-β-D-glucopyranosyl-(25*R*)-22-hydroxyl-5-ene-furostan-3β,26-diol-3-*O*-α-L- rhamnopyranosyl-(1→2)-[β-D-glucopyranosyl-(1→3)]-β-D-glucopyranoside
Gracillin	Diosgenin-3-*O*-α-L-rhamnopyranosyl-(1→2)-[β-D-glucopyranosyl-(1→3)]- β-D-glucopyranoside

progenin III. These properties of the crude enzyme from *A. oryzae* DLFCC-38 are also different from that of the previously reported pectinase hydrolyzing the 3-*O*-α-1,4-glycosides of steroidal saponins [4].

It is shown from the above results that the over 70 % of total steroidal saponins from *D. nipponica* Makino are protodioscin and dioscin, and when the total steroidal saponins were reacted with the enzyme from *A. oryzae* DLFCC-38, the main product was the progenin III. Thus, it is possible that the rare progenin III can be produced from the total steroidal saponins of *D. nipponica* Makino using the crude enzyme from *A. oryzae* DLFCC-38. To prepare rare progenin III from the major steroidal saponins of *D. nipponica* Makino efficiently, the crude enzyme production, reaction conditions, and process for progenin III production were studied in the following experiments.

Optimization of enzyme production

To obtain the optimal culture conditions, the effect of shaking speed, temperature, malt extract concentration, culture time, and enzyme inducer concentration on enzyme production by *A. oryzae* DLFCC-38 were investigated. When the strain was cultured at different shaking speeds: 100, 120, 150, and 200 rpm, the good enzyme production (17.8 U/ml) was observed at 150 rpm, but the low enzyme production was observed at 100, 120, and 200 rpm. The effect of culture temperature on enzyme production was studied at different temperature (25, 30, and 37 °C), and the temperature significantly affected the enzyme production. The highest enzyme activity (18.0 U/ml) was observed at 30 °C, but the enzyme activity was 8.5 U/ml at 25 °C, and 11.6 U/ml at 37 °C, respectively. When the strain was cultured in the medium containing different concentrations of malt extract [1, 3, 5, 7, and 9 % (w/v)], the good enzyme production (17.6 U/ml) was observed in the medium containing 5 % (w/v) malt extract.

The effect of the enzyme inducer (*D. nipponica* Makino extract) concentration on enzyme production was studied at different concentrations of *D. nipponica* Makino extract [0.0, 0.5, 1.0, 1.5, 2.5, 3.0, 3.5, and 4.0 % (v/v)]. As shown in Fig. 3, the good enzyme production was obtained with 2 % (v/v) *D. nipponica* Makino extract as inducer. However, the enzyme hydrolyzing steroidal saponins almost could not be produced without extract of *D. nipponica* Makino. Therefore, the enzyme hydrolyzing steroidal saponins is an inducible enzyme induced by the steroidal saponins in the extract of *D. nipponica* Makino. Summarizing the above optimal conditions for the enzyme production by *A. oryzae* DLFCC-38, the strain was cultured at 30 °C and 150 rpm in the medium containing 5 % (w/v) malt extract and 2 % (v/v) extract of *D. nipponica* Makino (enzyme inducer) to give a good enzyme production.

The dynamic change of the enzyme production by *A. oryzae* DLFCC-38 was examined at different culture times. As shown in Fig. 4, the cell growth increased rapidly from 12 to 60 h and reached a maximum after being cultured for 60 h, but decreased from 72 to 120 h. The enzyme production increased rapidly from 36 to 60 h, and the highest enzyme production of 18.1 U/ml was obtained after cultured for 72 h. Thus, the good enzyme production was obtained by *A. oryzae* DLFCC-38 cultivation in the medium containing 5 % (w/v) malt extract and 2 % (v/v) *D. nipponica* Makino extract at 30 °C for 72 h with shaking (150 rpm).

Optimization of enzymatic reaction conditions

The effects of pH and temperature on yield of progenin III by the crude enzyme from *A. oryzae* DLFCC-38 were investigated, respectively. As shown in Fig. 5, the optimal condition yielded the maximum production of progenin III was observed at pH 5.0 and 50 °C. The crude enzyme was stable at pH from 4.0 to 5.0. The progenin III yield above

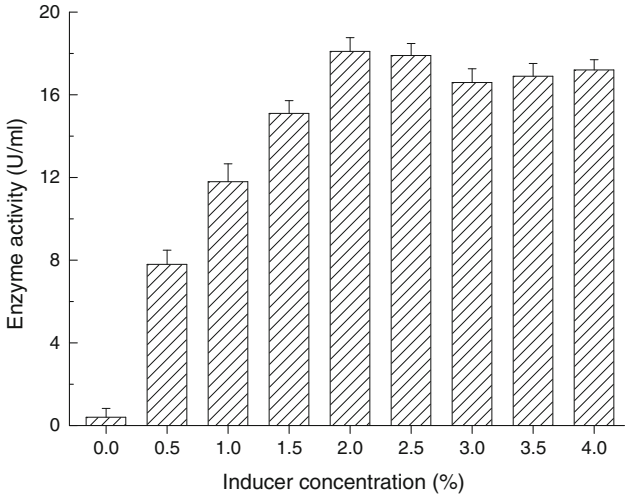

Fig. 3 Effect of enzyme inducer concentration on enzyme production by *A. oryzae* DLFCC-38. Cultivation was carried out in 5 % (w/v) malt extract medium containing various concentrations of *D. nipponica* Makino extract [0.0, 0.5, 1.0, 1.5, 2.5, 3.0, 3.5, and 4.0 % (v/v)] at 30 °C, 150 rpm for 72 h. Data represents the mean ± standard deviation of three independent experiments with three replicates each

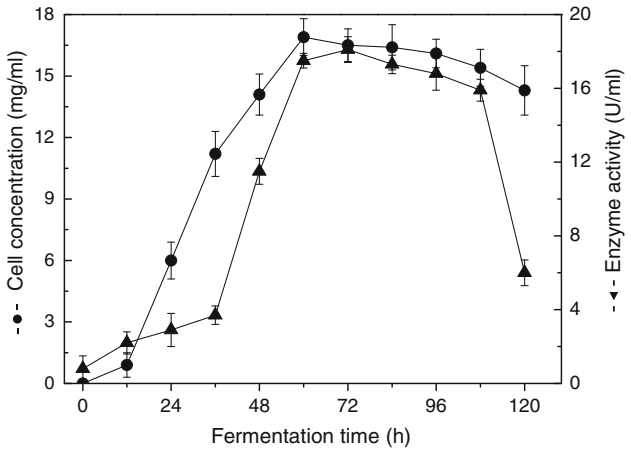

Fig. 4 Enzyme production during *A. oryzae* DLFCC-38 growth. Cultivation was carried out in the medium containing 5 % (w/v) malt extract and 2 % (v/v) *D. nipponica* Makino extract at 30 °C with shaking (150 rpm) for various periods of time. *Filled circle* cell concentration (mg/ml); *Filled triangle* enzyme activity (U/ml). Data represents the mean ± standard deviation of three independent experiments with three replicates each

35 % was obtained at temperature between 30 and 60 °C, but it decreased to 20 % at 70 °C. The metal ions such as K^+, Na^+, Ca^{2+}, Mg^{2+}, Zn^{2+}, and Cu^{2+} had no influence on the enzyme activity, but the enzyme activity was significantly inhibited by Fe^{3+}. In the presence of 50 mg/ml Fe^{3+}, the progenin III could not be detected in reaction mixture after reacted for 9 h.

The effect of substrate concentration on the yield of progenin III was investigated at different concentrations of total steroidal saponins. As shown in Fig. 6, the biotransformation period reaching the maximum progenin III was both below 9 h at total steroidal saponins concentration of 5–30 mg/ml. For concentrations of 5 and 10 mg/ml, the reaction time required to reach the maximum progenin III production was below 3 h. For concentrations of 15–30 mg/ml, the maximum progenin III production was obtained after reaction for 9 h. The maximum progenin III yield above 43.2 % was obtained at the substrate (total steroidal saponins) concentration of 5–20 mg/ml, whereas the maximum progenin III yield was 38.2 % at 25 mg/ml, and 35.3 % at 30 mg/ml, respectively (Fig. 6). The above results suggest that the substrate (total steroidal saponins) at a concentration below 20 mg/ml could be efficiently

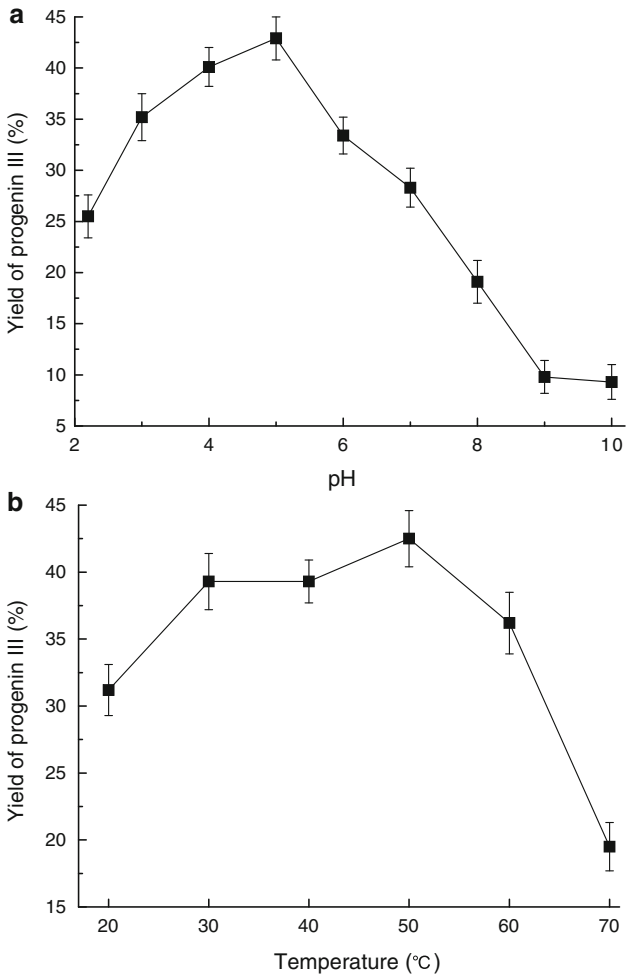

Fig. 5 Effects of pH (**a**) and temperature (**b**) on biotransformation of total steroidal saponins. Enzyme, 17.9 U/ml; substrate, total steroidal saponins (20 mg/ml in reaction mixture). **a** Reactions performed at different pH for 9 h at 50 °C. **b** Reactions performed at different temperatures for 9 h at pH 5.0. Data represents the mean ± standard deviation of three independent experiments with three replicates each

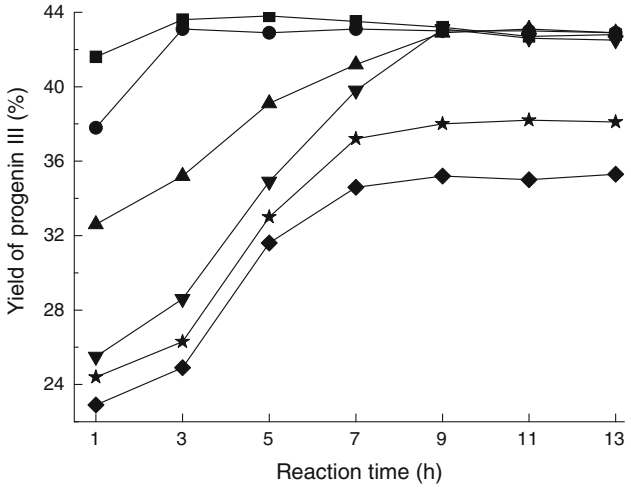

Fig. 6 Effect of substrate concentration on biotransformation of total steroidal saponins. The crude enzyme (17.9 U/ml) from *A. oryzae* DLFCC-38 was reacted with total steroidal saponins at final concentrations of 5 (*filled square*), 10 (*Filled circle*), 15 (*filled triangle*), 20 (*inverted filled triangle*), 25 (*filled star*), and 30 (*filled diamond*) mg/ml, respectively. The reaction was carried out at 50 °C and pH 5.0. Values presented are the average of three independent experiments with three replicates each

converted into progenin III by the crude enzyme from *A. oryzae* DLFCC-38, but the bioconversion was inhibited by high substrate concentrations (above 25 mg/ml). Therefore, the suitable substrate (total steroidal saponins) concentration was 20 mg/ml during the enzymatic conversion of the total steroidal saponins into progenin III.

Scale-up preparation of progenin III

Two liters of the crude enzyme was reacted with the same volume of 40 mg/ml total steroidal saponins solution (final substrate concentration of 20 mg/ml in reaction mixture) in the batch reactor, and the batch reaction was carried out twice. After being reacted for 9 h, the substrate of total steroidal saponins was almost completely biotransformed to major progenin III, and the produced product was precipitate in the reaction solution. The precipitate product was separated by filtration, and dried to obtain 117 g of crude progenin III whose purity was 56.3 % based on the TLC analysis. The progenin III yield was about 41.2 % from 160 g substrate. Then the crude progenin III was further separated using silica gel column, and 60.3 g of progenin III with at least 93.4 % purity was obtained from the 117 g crude progenin III.

To caution, the structure of this final product (progenin III of 60.3 g) was further identified by the method of NMR, the ^{13}C spectral data were shown in Table 1. Basing on the NMR spectra of ^{1}H, ^{13}C, DEPT, COSY, HSQC, and HMBC, the product was confirmed to be progenin III, i.e., diosgenin-3-*O*-α-L-rhamnopyranosyl-(1→2)-β-D-glucopyranoside, and its structure was shown in Fig. 2. The ^{13}C spectral data were also the same as those of previously reported in the literature [7, 11, 37] (Table 1).

Conclusions

The preparation of rare progenin III from total steroidal saponins of *Dioscorea nipponica* Makino by crude enzyme from *A. oryzae* DLFCC-38 was reported for the first time. The crude enzyme selectively transformed total steroidal saponins of *Dioscorea nipponica* Makino to major progenin III with a high yield, and the progenin III with at least 93.4 % purity was obtained by silica gel column chromatography. This study may be highly applicable for the practical preparation of progenin III for medicinal purposes.

Acknowledgments We thank Mr. Y.J. Wu, National Analytical Research Center of Electrochemistry and Spectroscopy (NARCES, P.R. China), for the NMR experiments. This work was supported by the Program for Liaoning Innovative Research Team in University (LNIRT: 2009T007) and the National Science of Foundation of P.R. China (NSFC).

References

1. Beit-Yannai E, Ben-Shabat S, Goldschmidt N, Chapagain BP, Liu RH, Wiesman Z (2011) Antiproliferative activity of steroidal saponins from *Balanites aegyptiaca*—an in vitro study. Phytochem Lett 4:43–47
2. Chien MH, Ying TH, Hsieh YS, Chang YC, Yeh CM, Ko JL, Lee WS, Chang JH, Yang SF (2012) *Dioscorea nipponica Makino* inhibits migration and invasion of human oral cancer HSC-3 cells by transcriptional inhibition of matrix metalloproteinase-2 through modulation of CREB and AP-1 activity. Food Chem Toxicol 50:558–566
3. Dong M, Feng XZ, Wu LJ, Wang BX, Ikejima T (2001) Two new steroidal saponins from the rhizomes of *Dioscorea panthaica* and their cytotoxic activity. Planta Med 67:853–857
4. Feng B, Huang HZ, Zhou WB, Kang LP, Zou P, Liu YX, Yu HS, Han BQ, Li YY, Zhang LL, Zhang T, Ma BP (2010) Substrate specificity, purification and identification of a novel pectinase with the specificity of hydrolyzing the α-1,4-glycosyl residue in steroidal saponin. Process Biochem 45:1383–1392
5. Feng B, Ma BP, Kang LP, Xiong CQ, Wang SQ (2005) The microbiological transformation of steroidal saponins by *Curvularia lunata*. Tetrahedron 61:11758–11763
6. Fu YY, Yu HS, Tang SH, Hu XC, Wang YH, Liu B, Yu CX, Jin FX (2010) New dioscin-glycosidase hydrolyzing multi-glycosides of dioscin from *Absidia* strain. J Microbiol Biotechn 20:1011–1017
7. Hu K, Dong AJ, Yao XS, Kobayashi H, Iwasaki S (1996) Antineoplastic agents; I. three spirostanol glycosides from Rhizomes of *Dioscorea collettii var. hypoglauca*. Planta Med 63:573–575
8. Hu K, Dong AJ, Yao XS, Kobayashi H, Iwasaki S (1997) Antineoplastic agents II: four furostanol glycosides from rhizomes of *Dioscorea collettii* var. *hypoglauca*. Planta Med 63:161–165

9. Huang W, Zhao HZ, Ni JR, Zuo H, Qiu LL, Li H (2006) The best utilization of *D. zingiberensis* C. H. Wright by an eco-friendly process. Bioresour Technol 99:7407–7411
10. Jin FX (2009) Biotransformation of natural products (Tianranchanwu Shengwu-zhuanhua in Chinese), Chemical Industry Press, Beijing, pp 54–69; 177–194
11. Jin J, Liu X, Teng R, Yang C (2002) Enzymatic degradation of Parvifloside. Acta Bot Sin 44:1243–1249
12. Kang TH, Moon E, Hong BN, Choi SZ, Son M, Park JH, Kim SY (2011) Diosgenin from *Dioscorea nipponica* ameliorates diabetic neuropathy by inducing nerve growth factor. Biol Pharm Bull 34:1493–1498
13. Kawasaki T, Yamauchi T (1968) Structures of prosapogenin-B and -A of dioscin and cooccurrence of B with dioscin in the Rhizoma of *Dioscorea Tokoro* Makino. Chem Pharm Bull 16:1070–1075
14. Kwon CS, Sohn HY, Kim SH, Kim JH, Son KH, Lee JS, Lim JK, Kim JS (2003) Anti-obesity effect of *Dioscorea nipponica* Makino with lipase-inhibitory activity in rodents. Biosci Biotechnol Biochem 67:1451–1456
15. Lei J, Niu H, Li T, Huang W (2012) A novel β-glucosidase from *Aspergillus fumigates* releases diosgenin from spirostanosides of *Dioscorea zingiberensis* C. H. Wright (DZW). World J Microbiol Biotechnol 28:1309–1314
16. Lin SH, Wang DM, Yang DP, Yao JH, Tong Y, Chen JP (2007) Characterization of steroidal saponins in crude extract from *Dioscorea nipponica* Makino by liquid chromatography tandem multi-stage mass spectrometry. Anal Chim Acta 599:98–106
17. Liu CZ, Zhou HY, Yan Q (2007) Fingerprint analysis of *Dioscorea nipponica* by high-performance liquid chromatography with evaporative light scattering detection. Anal Chim Acta 582:61–68
18. Liu HW, Hu K, Zhao QC, Cui CB, Kobayashi H, Yao XS (2002) Bioactive saponins from *Dioscorea futschauensis*. Pharmazie 57:570–572
19. Liu HW, Kobayashi H, Qu GX, Yao XS (2001) A new spirostanol saponin from *Dioscorea futschauensis*. Chin Chem Lett 12:613–616
20. Liu L, Dong YS, Qi SS, Wang H, Xiu ZL (2010) Biotransformation of steriodal saponins in *Dioscorea zingiberensis* C. H. Wright to diosgenin by *Trichoderma harzianum*. Appl Microbiol Biotechnol 85:933–940
21. Liu ZR, Zou WJ, Wang RZ, Zhou ZZ (2004) Clinic application of Di'ao Xin Xue Kang capsule for ten years in China. Chin J Tradit Chin Med Pharm 19:620–622
22. Man SL, Gao WY, Zhang YJ, Huang LQ, Liu CX (2010) Chemical study and medical application of saponins as anticancer agents. Fitoterapia 81:703–714
23. Man SL, Gao WY, Zhang YJ, Wang JY, Zhao WS, Huang LQ, Liu C (2010) Qualitative and quantitative determination of major saponins in *Paris* and *Trillium* by HPLC-ELSD and HPLC-MS/MS. J Chromatogr B 878:2943–2948
24. Mimaki Y, Yokosuka A, Kuroda M, Sashida Y (2001) Cytotoxic activities and structure-cytotoxic relationships of steroidal saponins. Biol Pharm Bull 24:1286–1289
25. Niño Osorio J, Mosquera Martinez OM, Correa Navarro YM, Mimaki Y, Sakagami H, Sashida Y (2003) Diospolysaponin A, a new polyoxygenated spirostanol saponin from the tubers of *Dioscorea polygonoides*. Heterocycles 60:1709–1715
26. Qian S, Yu HS, Zhang CZ, Lu MC, Wang HY, Jin FX (2005) Purification and characterization of dioscin-α-L-rhamnosidase from pig liver. Chem Pharm Bull 53:934–937
27. Quan B, Ma BP, Feng B, Xiong CQ, Wang YZ, Kang LP, Zhang J, Yan HL (2006) The microbiological transformation of protodioscin by *Aspergillus oryzae*. Chin J Nat Med 4:377–381
28. Rao AV, Gurfinkel DM (2000) The bioactivity of saponins: triterpenoid and steroidal glycosides. Drug Metabol Drug Interact 17:211–235
29. Sautour M, Mitaine-Offer AC, Lacaille-Dubois MA (2007) The *Dioscorea* genus: a review of bioactive steroid saponins. J Nat Med 61:91–101
30. Sautour M, Mitaine-Offer AC, Miyamoto T, Dongmo A, Lacaille-Dubois MA (2004) A new steroidal saponin from *Dioscorea cayenensis*. Chem Pharm Bull 52:1353–1355
31. Sautour M, Miyamoto T, Lacaille-Dubois MA (2006) Steroidal saponins and flavan-3-ol glycosides from *Dioscorea villosa*. Biochem Syst Ecol 34:60–63
32. Sparg SG, Light ME, Van Staden J (2004) Biological activities and distribution of plant saponins. J Ethnopharmacol 94:219–243
33. Tong QY, Qing Y, Shu D, He Y, Zhao YL, Li Y, Wang ZL, Zhang SY, Xing ZH, Xu C, Wei YQ, Huang W, Wu XH (2011) Deltonin, a steroidal saponin, inhibits colon cancer cell growth in vitro and tumor growth in vivo via induction of apoptosis and antiangiogenesis. Cell Physiol Biochem 27:233–242
34. Wang T, Choi RCY, Li J, Bi CWC, Zang L, Liu Z, Dong TTX, Bi K, Tsim KW (2010) Antihyperlipidemic effect of protodioscin, an active ingredient isolated from the rhizomes of *Dioscorea nipponica*. Planta Med 76:1642–1646
35. Wang T, Choi RCY, Li J, Bi CWC, Ran W, Chen X, Dong TTX, Bi K, Tsim KWK (2012) Trillin, a steroidal saponin isolated from the rhizomes of *Dioscorea nipponica*, exerts protective effects against hyperlipidemia and oxidative stress. J Ethnopharmacol 139:214–220
36. Wang YB, Zhang YC, Zhu ZY, Zhu SL, Li YX, Li M, Yu B (2007) Exploration of the correlation between the structure, hemolytic activity, and cytotoxicity of steroid saponins. Bioorg Med Chem 15:2528–2532
37. Watanabe Y, Sanada S, Ida Y, Shoji J (1983) Comparative studies on the constituents of ophiopogonis tuber and its congeners. II: studies on the constituents of the subterranean part of *Ophiopogon planiscapus* Nakai (1). Chem Pharm Bull 31:3486–3495
38. Xiao J, Wang NL, Sun B, Cai GP (2010) Estrogen receptor mediates the effects of pseudoprotodiocsin on adipogenesis in 3T3-L1 cells. Am J Physiol Cell Physiol 299:C128–C138
39. Yang SL, Ma YH, Liu XK (2005) Steroidal constituents from *Dioscorea parviflora* (article in Chinese). Yao Xue Xue Bao 40:145–149
40. Yin J, Kouda K, Tezuka Y, Le Tran Q, Miyahara T, Chen YJ, Kadota S (2003) Steroidal glycosides from the rhizomes of *Dioscorea spongiosa*. J Nat Prod 66:646–650
41. Zhang LJ, Yu HS, Kang LP, Feng B, Quan B, Song XB, Ma BP, Kang TG (2012) Two new steroidal saponins from the biotransformation product of the rhizomes of *Dioscorea nipponica*. J Asian Nat Prod Res 14:640–646
42. Zhang T, Liu H, Liu XT, Xu DR, Chen XQ, Wang Q (2010) Qualitative and quantitative analysis of steroidal saponins in crude extracts from *Paris polyphylla* var. *yunnanensis* and *P. polyphylla* var. *chinensis* by high-performance liquid chromatography coupled with mass spectrometry. J Pharm Biomed Anal 51:114–124
43. Zhu YL, Huang W, Ni JR, Liu W, Li H (2010) Production of diosgenin from *Dioscorea zingiberensis* tubers through enzymatic saccharification and microbial transformation. Appl Microbiol Biotechnol 85:1409–1416