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Article

Biotransformation of geniposide in *Gardenia jasminoides* to genipin by *Trichoderma harzianum* CGMCC 2979

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ABSTRACT

Trichoderma harzianum (T. harzianum) CGMCC 2979 was used to transform the geniposide in *Gardenia jasminoides* (G. jasminoides) to genipin, dispensing the use of purified enzyme and the extraction of geniposide from the raw material. At 30 °C, pH 6.1, and an initial G. jasminoides concentration of 80 g dried fruit per liter of medium, the geniposide-to-genipin conversion rate reached 97.8% after 48 h of fermentation. The genipin was purified from the fermentation broth by a combined method of XAD-16N-resin and silica-gel chromatography, yielding a total recovery of 62.3%. A 74.4-kDa geniposide- β -glucosidase implicated in the transformation of geniposide to genipin was purified from T. harzianum CGMCC 2979. It had optimum activity at 50 °C and pH 4.0–5.0. The K_m and V_{max} of the enzyme for geniposide were 3.6 mmol/L and 775 μ mol/h/mg protein, respectively. The simple, direct, and efficient biotransformation of geniposide in G. jasminoides to genipin by T. harzianum CGMCC 2979 that is described in this study could represent an alternative and effective method for producing genipin.

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1. Introduction

Genipin and its glycoside, geniposide, constitute the chief active components of the fruits of *Gardenia jasminoides* (G. jasminoides). These plants have been used in traditional Chinese medicine to treat inflammation, jaundice, and hepatic diseases. Geniposide is thought to achieve most of its biological activities through genipin [1]. Recent studies have demonstrated that genipin possesses numerous pharmacological activities, such as protection against oxidative damage, as well as hypoglycemic, hepatoprotection, cytotoxic, anti-inflammatory, and fibrinolytic activities [2–4]. Genipin has also become a compound of interest because it is a naturally occurring cross-linking agent, allowing it to form networks with important biological polymers,

such as gelatin, collagen, and chitosan. Genipin exhibits a cross-linking property comparable to chemical cross-linking agents such as glutaraldehyde, but with a toxicity that is about 10000 times less. Genipin has been regarded as a potential substitute for synthetic cross-linkers in the preparation of scaffolds for tissue engineering and drug carriers for enhanced delivery [5–7]. In addition, genipin is colorless, but it can react spontaneously with amino acids or some proteins to form dark blue pigments with luminescence characteristics, and this property of genipin has enabled its usage as a potential finger-mark reagent [8].

The enormous demand for genipin means that its production requires a simple and low-cost process. However, the content of genipin in gardenia fruits is rather low (about

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0.005%–0.01%), although the content of geniposide is high (about 3%–8%). Direct extraction of genipin from gardenia fruits through the use of acid hydrolysis is not suitable because iridoid glycosides are unstable in the presence of acid. The current production of genipin usually involves the use of an enzyme or microorganism to transform geniposide to genipin. Gong et al. [9] isolated a β -glucosidase, which displayed avid affinity and high catalytic efficiency for geniposide, from *Aspergillus niger* Au0847. Winotapun et al. [10] used commercial cellulase to catalyze simultaneous cell disruption and conversion of released geniposide to genipin. Fu et al. [11] transformed pure geniposide with *Aspergillus awamori* FYS-9, and the conversion of geniposide could reach 97.7%. However, in such methods, pure enzyme or geniposide need to be purified or purchased, which leads to a complex and expensive process. Furthermore, some commercial β -glucosidases have been reported to react with genipin to form blue pigment that displays a maximum absorbance around 580 nm without necessarily achieving a high yield of genipin [12,13].

Some attempts have been made to improve the method and reduce the cost of genipin preparation. Direct biotransformation of the raw herb by microorganisms, a new method that has the benefit of low cost and simple operation, has been successfully used in our laboratory to transform piceid in *Polygonum cuspidatum* to resveratrol using *Aspergillus oryzae* and to transform steroidal saponins in *Dioscorea zingiberensis* CH Wright to diosgenin using *A. oryzae* and *Trichoderma harzianum* (*T. harzianum*) [14,15]. Although the production of genipin from geniposide via biotransformation of the raw herb by *Penicillium nigricans* has been reported for *G. jasminoides*; with a geniposide-to-genipin conversion rate as high as 95%, the fermentation time is still too long (108 h) [16]. Furthermore, the conversion rate in that particular study was calculated from the reduction of geniposide, and no information was given regarding the yield of genipin in the biotransformation. This information is important for evaluating the practicality of the method. In addition, the study did not provide any information regarding the enzymes involved in the transformation. It is necessary to study the key enzymatic reaction associated with the biotransformation of the raw herb because understanding the mechanism of the biotransformation will help us design a better system.

During the course of screening for a fungal strain capable of converting geniposide to genipin in *G. jasminoides*, *T. harzianum* CGMCC 2979 was identified as the strain that could produce the highest level of genipin by direct biotransformation of the raw herb [16]. In this paper, the fermentation conditions for *T. harzianum* CGMCC 2979 were optimized and the purification of genipin from the fermentation broth was described (Scheme 1). In addition, a specific geniposide- β -glucosidase produced by *T. harzianum* during the fermentation process was also isolated, purified, and characterized.

2. Experimental

2.1. Materials and chemicals

All experiments were performed in triplicate. Data shown

Scheme 1. Biotransformation of geniposide to genipin by *Trichoderma harzianum* CGMCC 2979.

are the means of the triplicate experiments.

The dry fruits of *G. jasminoides* (DFA) were purchased from Anguo (Baoding, China). The fruits were ground into a powder, passed through a 60-mesh sieve, and then used directly for biotransformation. Geniposide and genipin standards (purities > 98.5%) were obtained from the National Pharmaceutical Engineering Center for Solid Preparation in Chinese Herbal Medicine (Nanchang, China). Protein markers for SDS-PAGE were purchased from Takara Co. (Otsu, Japan). Zingiberenis newsaponin, deltonin, diosgenin-triglucoside, and diosgenin-diglucoside standards (purities > 98%) were prepared as described previously [17]; diosgenin standard (purity > 98%) was obtained from Sigma Chemical Co. (St. Louis) and trillin (purity > 98%) was obtained from Wuhu Delta Pharmaceutical Co. (Wuhu, China). Polymeric adsorbent XAD-16N and silica gel were the products of Rohm and Hass Co. (Philadelphia) and Qingdao Haiyang Chemical Co. (Qingdao, China), respectively. DEAE Sepharose™ and Superdex 200 were purchased from GE Healthcare Biosciences (Pittsburgh). *p*-Nitrophenyl β -D-glucopyranoside (PNPG) was obtained from Shanghai Baoman Life Technology Co. Ltd (Shanghai, China). HPLC-grade acetonitrile was purchased from TEDIA (Cincinnati). All other chemicals used were of analytical grade. *T. harzianum* used as the transformation strain was previously isolated from a soil sample obtained locally. The organism is now preserved in the China General Microbiological Culture Collection Center (Beijing, China) with the number CGMCC 2979.

2.2. Analytical methods

HPLC was carried out using a Waters 600E Multi-solvent Delivery System connected to a 7725i injector and coupled to a UV detector (Model 2487, Waters Corporation, Milford). Analysis of geniposide and genipin was performed with a Sunfire C₁₈ column (150 mm $\times$ 4.6 mm $\times$ 5 μ m, Waters Corporation) at room temperature. The compounds were eluted from the column with a solution of acetonitrile/water (15:85 v/v) at a flow rate of 1 mL/min and detected by absorbance at 238 nm. The retention times of geniposide and genipin were 6.14 and 12.45 min, respectively. The steroidal saponins and their transformation products were analyzed by an established method [18]. The separation of steroidal saponins was performed on the same column as geniposide and genipin using acetonitrile (A) and water (B) as the mobile phase under the following gradient program with a flow rate of 1.0 mL/min:

0–14 min, 44% A→75% A; 14–15 min, 75% A→91% A; 15–32 min, 91% A. The column effluent was monitored at the UV wavelength of 203 nm, and the retention times of zingiberenis newsaponin, deltonin, diosgenin-triglucoside, diosgenin-diglucoside, trillin, and diosgenin were 7.40, 8.95, 10.10, 11.87, 15.40, and 30.04 min, respectively.

The concentration of protein was measured by the Bradford method using bovine serum albumin as the standard. β -Glucosidase activity was determined by a colorimetric method using PNPg as a substrate [19]. One unit of enzyme activity was defined as the amount of enzyme that liberates 1 μ mol of *p*-nitrophenol per hour. β -Glucosidase activity toward geniposide was determined as follows: 0.2 mL of a solution of 1 mg/mL geniposide was incubated with 0.2 mL enzyme solution for 2 h, and the sample was boiled for 5 min to stop the reaction. The reaction product was detected by HPLC as described above. The molecular weight of the enzyme was estimated by SDS-PAGE and further determined by mass spectrometry using a MALDI/TOF spectrometer (MALDI micro MX, Waters Corporation). The MS and NMR spectra were collected by a triple quadrupole mass spectrometer (Finnigan TSQ, Thermo Electron Corporation, Waltham) and 400M NMR Spectrometer (INOVA 400, Varian Corporation, Palo Alto), respectively.

2.3. Extraction of geniposide and genipin

About 3.0 g DFA powder was accurately weighed and separately extracted with 100 mL of each aqueous ethanol solution (0, 20%, 50%, 70%, and 100%) at ambient temperature for 12 h. Soxhlet extraction of DFA powder with 20% aqueous ethanol was also carried out over a period of 4 h. Each extract was filtered and analyzed by HPLC. In order to verify that the extraction rate of genipin at the optimal ethanol concentration (established for the extraction of DFA powder), about 4 mg standard genipin was accurately weighed and dissolved in 1 mL ethanol solution, and the concentration of genipin was analyzed by HPLC to calculate the extraction rate of genipin.

2.4. Culture and optimization of culture conditions

Unless indicated, all liquid cultures were prepared in 250-mL Erlenmeyer flasks. Well-developed fungal spores were collected from *T. harzianum* CGMCC 2979 grown on potato dextrose agar, and suspended in sterile water. The spore suspension was inoculated into 50 mL medium containing 40 g/L DFA. The culture was incubated on a rotary shaker at 30 °C and 150 r/min for 24 h, and 2 mL of this seed culture was used to inoculate 50 mL fresh fermentation medium. The fermentation medium contained only DFA plus water or DFA plus sodium phosphate buffer with a DFA concentration of 40 g/L. Both the seed medium and fermentation medium were sterilized at 121 °C for 20 min before inoculation to prevent microorganism contamination from DFA. Every 4 h, 1 mL of culture was sampled, and cells were diluted in 0.4% trypan blue solution and counted in a hemocytometer to assess the rate of dead blue cells from the total number of cells counted. The β -glucosidase activity of the culture was measured, and the colony morphology

of the culture was also compared with that of a pure culture of *T. harzianum* CGMCC 2979. After 96 h of incubation, 12.5 mL of ethanol was added to the fermentation broth, and the mixture was placed at room temperature for 12 h. The mixture was then filtered, and the filtrate was subjected to HPLC analysis.

To maximize the yield of genipin, factors that may influence the conversion rate (including fermentation time, DFA concentration, pH, and fermentation temperature) were optimized. The fermentation time was set at 12 to 120 h. The effects of different concentrations of DFA (40, 60, 80, 100, and 120 g/L) and fermentation temperatures (25, 30, and 37 °C) were also investigated. The effect of pH was determined by adjusting the medium to the desired pH (pH 2.1 to 7.1) with 66.7 mmol/L phosphate buffer.

The yield of genipin and the percentage of conversion rate are given by the following formulae:

$$\begin{aligned} \text{Yield (mg/g)} &= \text{genipin content (mg)/DFA raw herb (g)} \quad (1) \\ \text{Conversion rate (\%)} &= \text{content of genipin (mol)/initial content} \\ &\quad \text{of geniposide (mol)} \times 100\% \quad (2) \end{aligned}$$

2.5. Separation and purification of genipin in the fermentation broth

To investigate the efficiency associated with the removal of residual geniposide in the product of biotransformation, the fermentation broth was collected after 45 h of fermentation. Ethanol was added to the fermentation broth to give a final concentration of 20% (v/v), and the mixture was centrifuged at $10000 \times g$ for 20 min to remove the biomass. The supernatant was collected and designated as the 45-h sample to distinguish it from another sample taken after 48 h of fermentation. The sample contained 2.4 mg/mL genipin and 0.9 mg/mL geniposide, as determined by HPLC analysis. It was then subjected to XAD-16N-resin and silica-gel chromatography.

The resins were soaked in 95% ethanol, shaken for 24 h, and then thoroughly washed with distilled water. The adsorption condition for XAD-16N chromatography was tested as follows: eight 10-mL aliquots of the above 45-h sample were adjusted to pH 2.0–9.0, and each was added to a separate 50-mL flask. To each flask, 1 g of hydrated XAD-16N resin was added, and the flasks were shaken at 130 r/min on a rotary shaker for 12 h at 25 °C. After adsorption, the liquid fraction was discarded; the resin was washed with distilled water, soaked in 10 mL 80% (v/v) ethanol, and shaken for another 12 h at the same temperature and speed. The ethanol solution was removed, and the concentrations of genipin and geniposide in the ethanol solution collected from each of the 8 flasks were measured to determine the amounts of genipin and geniposide desorbed from XAD-16N. The adsorption capacity (q_e) of XAD-16N was calculated according to the following equation [20]:

$$q_e = (C_0 - C_e) \times V_i / ((1 - M) \times W) \quad (3)$$

where C_0 is the initial concentration (mg/mL) of genipin, C_e is the concentration (mg/mL) of genipin at equilibrium, V_i is the volume of the initial sample solution (mL), M is the water content, which was 65% as determined by a published method [20], and W is the weight of resin (g).

The effect of the ethanol concentration on the ratio of genipin desorption was determined using a glass column (2.1 cm × 15 cm) packed with 45 g XAD-16N (wet resin). The bed volume of the resin was 50 mL. 100 mL of the 45-h sample was adjusted to pH 4.0 and then applied to the column. After the adsorption reached saturation, the column was washed with three bed volumes of distilled water and then eluted with a stepwise gradient of increasing ethanol concentrations (30%, 40%, 50%, 60%, 70%, 80%, 90%, and 100%) using 100 mL for each concentration. The eluent was monitored by HPLC.

The silica gel-chromatography step was carried out in a glass column (2.1 cm × 24 cm) packed with 40 g of silica gel. 30 mL of the 45-h sample was concentrated to dryness under vacuum, and about 50 mg of this residue was loaded onto the silica gel column. The column was eluted with solutions containing different ratios of chloroform to methanol (85:15, 84:16, 83:17, and 82:18, v/v), and the eluent was collected and monitored by HPLC.

The purification of genipin was also scaled up to 200 mL using fermentation broth taken after 48 h of fermentation, the time point at which the maximal geniposide-to-genipin conversion rate was detected. The sample was extracted with 50 mL ethanol for 12 h. After centrifugation at $10000 \times g$ for 20 min, 240 mL supernatant was recovered. The supernatant, which contained 2.6 mg/mL genipin and 0.09 mg/mL geniposide (as determined by HPLC), was designated as the 48-h sample. It was adjusted to pH 4.0 and then applied to a column (3.0 cm × 30 cm) packed with 145 g XAD-16N resin. The column was first washed with two bed volumes of 30% (v/v) ethanol in water (pH 4.0), followed by elution with two bed volumes of 60% (v/v) ethanol in water (pH 6.5). The eluent, which contained genipin as detected by UV absorbance, was concentrated to dryness under vacuum and loaded onto a column (4.0 cm × 25 cm) packed with 150 g silica gel. The column was eluted with 600 mL chloroform-methanol (83:17, v/v) solution. The eluent containing genipin was concentrated to dryness and then resuspended in 2.0 mL 66.7% methanol in water to give pure genipin.

2.6. Purification of β -glucosidase

Culture acquired from fermentation under the optimized conditions was centrifuged at $10000 \times g$ for 10 min to remove the cells. Solid $(\text{NH}_4)_2\text{SO}_4$ was slowly added to the supernatant while stirring to 75% saturation and was then stored at 4 °C overnight. The mixture was centrifuged at $10000 \times g$ for 10 min to collect the protein precipitate. This crude protein was dissolved in 66.7 mmol/L sodium phosphate buffer (pH 5.0), and the insoluble material was removed by centrifugation ($10000 \times g$ for 10 min). The supernatant was passed through a 0.45- μm membrane, and a portion of the pigment present in it was removed by ultrafiltration. The sample was loaded onto a DEAE Sepharose™ column (3.0 cm × 12 cm) pre-equilibrated with 66.7 mmol/L sodium phosphate buffer (pH 5.0) at a flow rate of 1 mL/min. The flow-through fraction was concentrated by ultrafiltration and then applied to a Superdex 200 column (3.0 cm × 21 cm) pre-equilibrated with 66.7 mmol/L sodium phosphate buffer (pH 5.0). The column was eluted with the

same buffer at a flow rate of 1 mL/min. All chromatographic runs were monitored for the presence of protein at 280 nm, and all of the fractions collected were assayed for β -glucosidase activity. Active fractions were pooled and stored at 4 °C for further analysis.

2.7. Enzyme properties

The effect of temperature on enzyme activity was determined for a temperature range of 20–70 °C, and the effect of pH was investigated with a pH range of 3.0–8.0 using 66.7 mmol/L sodium phosphate buffer adjusted to the desired pH. The visible spectrum from 500 to 800 nm was scanned to monitor the production of byproduct (blue pigment).

Substrate specificity was tested with geniposide from *G. jasminoides* and steroidal saponins from *D. zingiberensis*, including zingiberenis newsaponin, deltonin, diosgenin-triglucoside, diosgenin-diglucoside and trillin. Substrate solution was prepared by dissolving 1 mg geniposide or steroidal saponin in 1 mL 66.7 mM sodium phosphate buffer (pH 5.0), and 0.2 mL of the purified enzyme solution was then added to the same volume of substrate. For steroidal saponins, the reaction was carried out at 50 °C for 24 h. After that, 0.2 mL butanol was added to the reaction mixture and vortexed for 1 min, and the reaction product was then extracted with butanol and detected by HPLC. For geniposide, the reaction was also carried out at 50 °C, but for different periods of time (1.0, 1.5, 2.0, 2.5, and 3.0 h), and the reaction was terminated by boiling in water for 5 min.

The K_m and $V_{\max}$ values of the purified geniposide- β -glucosidase were determined from a Lineweaver-Burk plot of the substrate-saturation curve obtained with a geniposide concentration range of 1.18 to 2.95 mmol/L at pH 5.0 and 50 °C.

3. Results and discussion

3.1. Determination of geniposide content in DFA and effect of ethanol concentration on geniposide yield

When geniposide was extracted directly from DFA by Soxhlet's extraction, a content of 64.1 ± 1.5 mg/g was obtained, and this was taken as the content of the geniposide in DFA. To determine the contents of genipin and geniposide more efficiently, the effect of ethanol on the extraction yields of geniposide and genipin was also investigated. Because geniposide is mainly present in the solid matter of the fruit and genipin is secreted into the supernatant after biotransformation [16], DFA and standard genipin were selected as the materials for extraction. With 0, 20%, 50%, 70%, and 100% ethanol, the yields of geniposide from DFA were 55.4 ± 0.8 , 63.8 ± 2.4 , 61.9 ± 2.9 , 51.6 ± 0.5 , and 26.8 ± 1.3 mg/g DFA, respectively. The highest yield was obtained with 20% ethanol, and this was almost the same as the yield obtained by the Soxhlet extraction. The extraction of standard genipin achieved a rate of $99.4\% \pm 0.2\%$ when the concentration of genipin in the sample was 4 mg/mL and the concentration of ethanol was 20%. Samples that were taken at the pre-biotransformation and post-biotransformation stages were treated by adding ethanol to a final

concentration of 20% and then stored for further analysis.

3.2. Biotransformation of geniposide in *G. jasminoides* by *T. harzianum* CGMCC 2979

DFA powder was incubated with the fungus at 30 °C for 96 h with shaking at 150 r/min. A comparison of the content of substrate with that of product at the pre-biotransformation and post biotransformation stages showed that geniposide was transformed into genipin at a conversion rate of 93%. Thus, genipin could be obtained from the bioconversion of DFA, dispensing with the use of purified enzyme and extraction of geniposide from the raw material. During fermentation, β -glucosidase was secreted by *T. harzianum* CGMCC 2979 to hydrolyze the geniposide in DFA, producing glucose as one of the byproducts. The glucose produced was further used as a carbon source for the growth of the fungus, thereby reducing the feedback inhibition caused by glucose and increasing the efficiency of the biotransformation. To obtain a higher yield of genipin in a shorter period of time, the fermentation conditions were further optimized. The result of the temperature-effect experiment showed that, at the end of fermentation, the conversion rates of genipin obtained at 25, 30, and 37 °C were $92.6\% \pm 3.7\%$, $97.7\% \pm 0.9\%$, and $88.6\% \pm 2.2\%$, respectively. Various groups also have reported that 30 °C is the optimum temperature for culturing *T. harzianum* [15,21]. Thus, the production of genipin by *T. harzianum* appeared to be dependent on the growth conditions of the fungus.

The concentration of DFA in the culture also had a significant effect on the conversion rate (Fig. 1(a)). The conversion rate of genipin exceeded 95% for DFA concentrations ranging from 40 to 80 g/L. However, when the concentration of DFA exceeded 80 g/L, the conversion rate of genipin declined dramatically.

The pH of the medium also affected the conversion rate significantly. The conversion rate was less than 60% when the pH of the medium was kept at 2.1 to 5.1, but it reached $98.3\% \pm 3.1\%$ when the pH was increased to 6.1, and then the conversion rate decreased again when the pH was further increased to 7.1 (Fig. 1(b)). These data indicated that pH 6.1 was the best pH for the fungus to produce the enzyme needed to convert geniposide to genipin. The optimum conversion rate at pH 6.1

could also be attributed to the presence of sodium phosphate salt, which provided a source of phosphorus and acted as a buffering agent to stabilize the pH of the culture, facilitating both *T. harzianum* growth and enzyme secretion.

The optimized culture conditions were as follows: 80 g/L DFA at 30 °C and pH 6.1. No additional carbon or nitrogen source was necessary for the fermentation, which made the process simple and efficient. A time course experiment with the above conditions showed that the conversion rate of genipin increased quickly from 24 to 48 h; the concentration of geniposide was 13.2 mmol/L before biotransformation and decreased constantly as the fermentation progressed, leading to a dramatic decline after 40 h of fermentation. At 48 h, a maximum genipin level of 12.9 mmol/L or 3.0 g/L was attained, which was about 1.88 times higher than that obtained from *Penicillium nigricans* biotransformation (6.88 mmol/L, 1.6 g/L). The conversion rate and yield reached 97.8% and 3.6 mg/g DFA, respectively (Fig. 2). Therefore, 48 h was regarded as the best fermentation time for genipin production, and this was 55.6% shorter than the fermentation time stated in a previous study [16]. Beyond 48 h, the conversion rate decreased gradually ($91.4\% \pm 1.3\%$ at 50 h, $85.5\% \pm 4.8\%$ at 52 h, and $83.6\% \pm 3.3\%$ at 55 h).

The trypan blue exclusion experiment showed that the rate of fungal cell death was lower than 10.0% during the first 32 h, and then it increased to 13.0% at 40 h, 16.6% at 48 h, and finally to 25.0% at the end of the fermentation (55 h). The time course experiment showed that β -glucosidase activity was first detected at 12 h, reached a maximum at 40 h (23.77 U/mL), and then gradually decreased to 19.76 U/mL at 55 h (Fig. 2). The colony morphology of the culture was identical to that of the pure culture of *T. harzianum* CGMCC 2979. These data suggested that the β -glucosidase, which transformed geniposide to genipin in gardenia fruits, was produced by *T. harzianum* CGMCC 2979, and the decrease in β -glucosidase activity after 40 h of fermentation was partially caused by fungal cell death.

3.3. Separation and purification of genipin in the fermentation broth

The static adsorption experiment showed that genipin could be adsorbed to XAD-16N, and the adsorption capacity was af-

Fig. 1. Effects of dry fruits of *Gardenia jasminoides* (DFA) content (a) and medium pH (b) on the geniposide-to-genipin conversion rate.

Fig. 2. Time course biotransformation of geniposide (1) in *G. jasminoides* into genipin (2) by *T. harzianum* and β -glucosidase activity of the culture (3).

ected by the initial pH of the sample. When the initial pH of the supernatant increased from 2.0 to 7.0, the q_e of genipin for XAD-16N increased with pH up to 4.0, and then it decreased slightly with further increases in pH; when the pH was greater than 7.0, no genipin was detected in the eluent, suggesting that pH = 4 was the best for genipin to adsorb to XAD-16N. The q_e of geniposide remained constant at about 3.2 mg/g resin (Fig. 3(a)).

The best condition for eluting genipin from the XAD-16N resins was determined by eluting the adsorbed genipin with a stepwise gradient of increasing ethanol concentrations. Dynamic desorption curves showed that the genipin concentration in the eluent increased with increasing ethanol concentrations in the eluting solvent, reaching a maximum when the eluting solvent contained 50% (v/v) ethanol, but the concentration started to decrease when the ethanol concentration in the solvent was further increased. Because the geniposide was eluted by a low ethanol concentration (Fig. 3(b)), the column was first washed with 30% ethanol, and elution followed with 60% ethanol. The yields of genipin and geniposide were 92.2% and 32.7%, respectively. These data indicated that most of the

geniposide could not adsorb to XAD-16N in the presence of 20% (v/v) ethanol and pH 4.0, and it could be removed from the resin during the washing step, which used 30% (v/v) ethanol in water at pH 4.0.

Because of the complicated mixture of components in the fermentation broth, the separation of genipin and geniposide from the other components in the clarified broth (after extraction with ethanol) was also carried out by silica-gel chromatography. Genipin that was adsorbed to the silica gel was thoroughly eluted by 83:17 (v/v) methanol-chloroform, while the adsorbed geniposide was eluted by 82:18 (v/v) chloroform-methanol. Thus, 83:17 (v/v) chloroform-methanol was chosen as the best eluting solvent.

Genipin was successfully separated and purified from the fermentation broth that was collected at the point of the highest geniposide-to-genipin conversion rate (48 h). The separation and purification of genipin was achieved using the optimized XAD-16N-resin chromatography method combined with silica-gel chromatography. The unconverted geniposide, pigment, and other impurities in the fermentation broth were effectively removed, yielding 389.0 mg colorless genipin with a purity of 98.3% from 200 mL culture broth (by HPLC analysis). The yields of genipin that were achieved by the individual purification methods were 87.1% (XAD-N16) and 77.0% (silica gel), whereas the yield obtained from the combined method was 62.3%.

MS analysis of the purified product in negative mode produced two ion peaks, one at 225 and the other at 261, representing $[M-H]^-$ and $[M+Cl]^-$, respectively. Thus, the molecular weight of the purified product was 226, which was consistent with the molecular weight of genipin. The NMR data revealed the following: 1H NMR (CH_3OD) δ : 7.53 (s, H-3), 5.83 (s, H-7), 4.80 (d, $J = 8.4$ Hz, H-1), 4.32 (d, $J = 14.4$ Hz, H-10a), 4.22 (d, $J = 14.4$ Hz, H-10b), 3.70 (s, $-OCH_3$), 3.16 (m, H-5), 2.86 (1H, m, H-6b), 2.50 (1H, m, H-9), and 2.06 (1H, m, H-6a); ^{13}C NMR (400 MHz, CH_3OD) δ : 170.0 ($-CO_2-$), 154.4 (C-3), 145.7 (C-8), 128.4 (C-7), 111.9 (C-4), 97.9 (C-1), 61.8 (C-10), 51.8 ($-OCH_3$), 48.6 (C-9), 40.1 (C-6), and 37.8 (C-5). All of these findings were in accordance with those of genipin reported by Kim et al. [4].

To our knowledge, the biotransformation of *G. jasminoides*

Fig. 3. Separation and purification of genipin and geniposide by XAD-16N-resin chromatography. (a) Static adsorption capacity for genipin and geniposide; (b) Dynamic desorption curves for genipin (1) and geniposide (2).

by *T. harzianum* CGMCC 2979 achieved the shortest fermentation time, highest geniposide-to-genipin conversion rate, and highest concentration of genipin compared with other reported methods for genipin production via raw herb biotransformation. This biotransformation of *G. jasminoides* using *T. harzianum* CGMCC 2979, together with the high selectivity between genipin and geniposide in their adsorption to XAD-16N resin and silica gel, would offer an alternative method for producing genipin with high yield and high purity.

3.4. Purification and enzymatic characteristics of geniposide- β -glucosidase

To study the enzymatic characteristics of geniposide- β -glucosidase, the enzyme was purified by a method that combined ammonium sulfate precipitation with DEAE Sepharose™ chromatography and Superdex 200 chromatography. The flow-through at the DEAE Sepharose™ step contained β -glucosidase activity, while most of the pigment in the preparation remained bound to the column. Further chromatography of the unbound fraction on the Superdex-200 column yielded a purified enzyme, achieving a 16-fold purification and a yield of about 14.7%. Using PNPG as a substrate, the specific activity of the purified geniposide- β -glucosidase was determined to be 72.0 U/mg protein. The purification results are summarized in Table 1.

The molecular weight of the purified enzyme estimated by SDS-PAGE was 76.5 kDa (Fig. 4), but MALDI/TOF MS analysis gave a lower value, 74.4 kDa. This enzyme was smaller than the geniposide-hydrolyzing β -glucosidases that were isolated from a human intestinal anaerobe and *A. niger* Au0847, which have molecular weights of 90 and 230 kDa, respectively [9, 22].

The geniposide- β -glucosidase that was purified from *T. harzianum* showed maximum activity within the pH range of 4.0–5.0. The geniposide-to-genipin conversion rate was above 90% at pH 6.0, but it dropped to 70% at pH 7.0. The activity of this geniposide- β -glucosidase gradually increased with temperature, peaking at 50 °C and sharply decreasing at 60 °C. The enzyme lost all activity at 70 °C. A time course experiment showed that genipin appeared mainly after 40 h of fermentation (Fig. 2), and this was also the time at which the maximum level of β -glucosidase activity was detected. These data indicated that that 0–40 h was the period of enzyme production, and 40–48 h was the period of biotransformation. During the period of biotransformation, increasing the fermentation temperature will shorten the time of biotransformation and increase the produce yield [14]. The time course data, together with higher catalytic activity at 40–50 °C, suggested that the fermentation time of geniposide could be further shortened by

Fig. 4. SDS-PAGE of geniposide- β -glucosidase. Lane 1, purified geniposide- β -glucosidase; Lane 2, protein markers.

increasing the fermentation temperature to 40–50 °C during the period of biotransformation. This would improve the efficiency of the biotransformation.

The purified enzyme showed considerable activity toward geniposide, converting 91.0% and 98.9% of it to genipin, after 1 and 2 h of incubation at 50 °C and pH 5.0, respectively. The K_m and V_{max} values of the purified enzyme for geniposide were 3.6 mmol/L and 775 μ mol/h/mg protein, respectively. ($y = 0.00467x + 0.00129$, $R^2 = 0.9746$). The K_m value of the purified geniposide- β -glucosidase in this work was comparable to that from *A. niger* Au0847 (2.93 mmol/L) [9]. However, the purified enzyme could not hydrolyze the α -(1 $\rightarrow$ 2)-rhamnoside of zingiberis newsaponin and deltonin and the β -(1 $\rightarrow$ 3)- and β -(1 $\rightarrow$ 4)-glucosides of steroidal saponins, including zingiberis newsaponin, deltonin, diosgenin-triglucoside, and diosgenin-diglucoside. It could not hydrolyze trillin, which is also a mono β -1-4 glycosidic saponin with different aglycones. These data suggested that the purified enzyme was a specific geniposide- β -glucosidase, and it could probably recognize the aglycone of geniposide. A β -glucosidase from *T. harzianum* with a similar molecular weight (75 kDa) has also been reported, and the substrate specificity of the enzyme has been determined using aryl-glycosides, disaccharides, and some oligosaccharides as substrates. However, the catalytic activity of the enzyme with respect to natural products has not been reported [23].

The absorption spectra of the pre- and post-enzymatic hydrolysate at 500 to 800 nm were compared, and no distinct change was observed. These data suggested that the purified geniposide- β -glucosidase did not react with genipin. This property of the enzyme may be one of the reasons for the high geniposide-to-genipin conversion rate in the biotransformation of raw herb.

Table 1

Purification of geniposide- β -glucosidase.

Step	Total activity* (U)	Total protein (mg)	Specific activity (U/mg)	Yield (%)	Purification factor
NH ₄ SO ₄ precipitation	196.0	28.5	6.8	100	—
DEAE sepharose™	109.8	12.3	8.9	56.0	1.3
Superdex-200	28.8	0.4	72.0	14.7	10.6

* Activity was measured with PNPG as a substrate.

Graphical Abstract

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Biotransformation of geniposide in *Gardenia jasminoides* to genipin by *Trichoderma harzianum* CGMCC 2979

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We described a simple and effective method for preparing genipin based on the biotransformation of geniposide in *Gardenia jasminoides* by *Trichoderma harzianum*. The mechanism of biotransformation was also discussed.

4. Conclusions

We have described a simple and effective method for preparing genipin based on the biotransformation of geniposide in *G. jasminoides* by *T. harzianum* CGMCC 2979. Genipin produced from the biotransformation of geniposide was extracted from the fermentation broth (harvested after 48 h of fermentation, the optimum time of conversion) with XAD-16N resin and subsequently purified by silica-gel chromatography, yielding a total recovery of 62.3% and a purity of 98.3%. Compared with previously reported methods, this new method achieved the shortest fermentation time, highest geniposide-to-genipin conversion rate, and genipin concentration. In addition, a pure geniposide- β -glucosidase, which showed a high level of specific activity toward geniposide, was purified and characterized. This simple and efficient biotransformation of geniposide in *G. jasminoides* to genipin by *T. harzianum* CGMCC 2979 could be considered as a promising alternative method for practical production of genipin for various applications.

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哈茨木霉CGMCC 2979生物转化栀子中的京尼平苷制备京尼平

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摘要: 采用微生物直接转化药材的方法, 将栀子中的京尼平苷转化为京尼平, 无需糖苷酶和京尼平苷的制备。在培养温度为30 °C, pH 6.1以及栀子载量为80 g/L的条件下, 48 h京尼平苷的转化率为97.8%。转化后的京尼平通过XAD-16N大孔树脂偶联硅胶层析的方法, 制备得到纯度大于95%的京尼平, 收率为62.3%。在催化、转化机制研究中, 从哈茨木霉CGMCC2979的发酵液中分离得到了分子量为74.4 kDa的京尼平苷- β -葡萄糖苷酶, 该酶最优催化条件为50 °C和pH 4.0–5.0。 K_m 和 V_{max} 分别为3.6 mmol/L和775 μ mol/h/mg蛋白。本文提供了一种简便、高效制备京尼平的新方法。

关键词: 京尼平苷; 京尼平; 哈茨木霉; 生物转化; 京尼平苷- β -葡萄糖苷酶; 纯化。

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1. 前言

栀子作为一种传统的中药, 被广泛用于炎症和黄疸型肝炎的治疗。京尼平与其糖苷-京尼平苷是栀子果实的主要活性成分, 其中京尼平是最主要的活性成分, 京尼平苷通过转化为京尼平发挥作用^[1]。近年的研究^[2–4]表明京尼平具有广泛的药理活性, 如抗氧化、降血糖、保肝、细胞毒, 抗炎以及纤溶活性等; 同时它还是一种天然的交联剂, 备受关注。和其他化学交联剂(如戊二醛)相比, 京尼平的交联能力相当, 能够和很多重要的生物聚合物如明胶、胶原、壳聚糖交联, 但毒性要比戊二醛低10000倍, 所以京尼平也是一种合成交联剂的理想替代物, 在组织工程和药物载体领域发挥重要作用^[5–7]。此外, 京尼平虽是一种无色物质, 但是能够与氨基酸和一些蛋白形成发光的蓝黑色素, 京尼平的这个特征, 能够有望使之用于指纹鉴别领域^[8]。

京尼平需求量大, 要求其制备过程简便低廉。但是京尼平在栀子果实中的含量很低, 只占0.005%–0.01%, 而京尼平苷的含量却很高, 约占3%–8%。直接提取法并不适合从栀子中制备京尼平, 该方法需要利用酸将京尼平苷水解为京尼平, 但京尼平苷属环烯醚萜苷, 在酸中不稳定, 收率很低。目前制备京尼平的方法主要是用酶或者微生物水解京尼平苷的糖苷键, 使之转化为京尼平。Gong等^[9]从黑曲霉AU0847中分离得到了一高亲和性、高催化活性的 β -葡萄糖苷酶; Winotapum等^[10]用一种商业化的纤维素酶将植物细胞破壁、京尼平苷转化为京尼平整合为一步进行; Fu等^[11]使用灰绿曲霉转化纯京尼平苷, 转化率可达97.7%, 但是这些方法需要制备纯酶或者纯京尼平苷, 过程复杂, 成本高。而且一些商业化的 β -葡萄糖苷酶容易和京尼平反应生成最大波长为580 nm的蓝色素, 从而影响京尼平的收率^[12,13]。

微生物直接转化药材的新方法具有成本低、操作简

便的特点, 具有较好的应用前景, 本实验室已成功将其用于米曲霉转化虎杖制备白藜芦醇和利用米曲霉、哈茨木霉转化盾叶薯蓣制备薯蓣皂苷元中^[14,15], Xu等^[16]报利用*Penicillium nigrificans*转化京尼平苷制备京尼平, 转化率可达95%, 但发酵时间(108 h)较长, 京尼平苷的转化率是按照其减少量计算而得的, 且没有京尼平的收率信息, 而这些信息对于评估该方法的应用意义重大。同时, 该研究也没有给出转化中关键酶的信息, 它对于阐明生物转化的机理具有十分重要的意义。在筛选具有转化栀子中京尼平苷为京尼平活性的真菌实验中, 我们发现利用哈茨木霉CGMCC 2979对栀子中京尼平苷进行生物转化, 获得的京尼平含量是已知微生物转化药材研究中最高的^[16]。本文报道了哈茨木霉CGMCC 2979发酵(图示1)条件优化和京尼平分离纯化方法, 以及哈茨木霉CGMCC 2979产生的京尼平苷- β -葡萄糖苷酶的分离、纯化以及其酶学特征。

2. 实验部分

2.1. 材料和试剂

本文所有的实验都重复了三次, 数据是三次实验的平均值。

栀子干燥果实(DFA)购自河北省安国, 经粉碎过60目筛后用于生物转化。京尼平苷和京尼平的标准品购自中药固体制剂制造技术国家药物工程中心(南昌), 纯度大于98.5%。SDS-PAGE蛋白标准品购自宝生物(天津, 日本), 盾叶新苷、三角叶皂苷、薯蓣皂苷元-三葡萄糖苷、薯蓣皂苷元-二糖苷标准品由本实验室按照文献^[17]制备, 薯蓣皂苷元标准品购自Sigma公司(圣路易斯, 美国), 延龄草次苷购自芜湖delta制药公司(芜湖)。大孔吸附树脂XAD-16N和硅胶分别购自Rohmhaas公司(费城, 美国)和青岛海洋化工公司(青岛)。DEAE SepharoseTM和Superdex 200均购自GE healthcare biosciences公司(匹斯堡, 美国)。p-nitrophenyl- β -glucopyranoside (pNPG)购于

上海保曼生物技术公司(上海). 其它的化学品均是分析纯. 用于生物转化的*Trichoderma harzianum*菌株分离自本地土壤, 保存于中国普通微生物菌种保藏管理中心(北京), 编号为:CGMCC 2979.

2.2. 分析方法

HPLC(Waters公司, 米尔福德, 美国)由600E液体输送系统、7725i进样器, 2487型UV检测器组成. 京尼平苷和京尼平的分析使用Sunfire C₁₈柱(150 mm × 4.6 mm × 5 μm, 美国Waters公司)在室温下进行. 洗脱条件为乙腈/水(15:85 v/v), 流速1.0 mL/min, 检测波长为238 nm, 京尼平苷和京尼平的保留时间分别为6.14和12.45 min. 甾体皂苷的分析方法参见文献[18], 使用乙腈(A)和水(B)作为流动相, 流速为1.0 mL/min, 洗脱条件为: 0–14 min, 44% A → 75% A; 14–15 min, 75% A → 91% A; 15–32 min, 91% A. 检测波长为203 nm, 盾叶新苷、三角叶皂苷、薯蓣皂苷元-三葡萄糖苷、薯蓣皂苷元-二糖苷、延龄草次苷和薯蓣皂苷元的保留时间分别为7.40, 8.95, 10.10, 11.87, 15.4和30.04 min.

蛋白质浓度采用bradford法测定, 用牛血清白蛋白作为标准品; β-葡萄糖苷酶的活性采用对硝基苯(pNPG)为底物的比色法测定^[19], 1单位酶活定义为每小时释放1摩尔pNPG的酶量. β-葡萄糖苷酶对京尼平苷的活性按照如下方法测定: 取0.2 mL京尼平苷溶液(1 mg/mL), 和0.2 mL酶液培养2 h后, 将样品煮沸5 min终止反应, 反应产物由上述HPLC方法定量分析. 酶的分子量先由SDS-PAGE估算, 然后用MALDI/TOF确定(MALDI, microMXWaters, 美国热电, 沃尔瑟姆, 美国), 纯化后的京尼平由三重四极杆质谱(Finnigan, TSQ, 美国热电)和核磁共振仪(INOVA400, 瓦里安公司, 帕罗奥图, 美国)验证结构.

2.3. 提取京尼平苷和京尼平

精确称取约3.0 g的DFA粉末, 分别加入0, 20%, 50%, 70%或100%的乙醇水溶液100 mL, 室温静置12 h. 索氏提取DFA采用20%乙醇水溶液, 提取时间为4 h. 上述提取液过滤除去残渣后, 进行HPLC定量分析确定含量. 为了验证京尼平在优化乙醇浓度下的提取率, 精确称量4.0 mg京尼平标准品, 溶于1 mL优化的乙醇溶液中, 京尼平的含量也由HPLC测定.

2.4. 培养和培养条件优化

除非特别标明, 所有的液体培养均在250 mL三角瓶中进行, 哈茨木霉CGMCC 2979在PDA培养基上培养至孢子生长旺盛后, 溶于无菌水. 孢子悬液接入含有50 mL

40 g/L DFA的种子培养基中, 在摇床上150 r/min振荡培养24 h后, 取2 mL种子液加入到50 mL新鲜培养基中发酵培养. 发酵培养基只含有DFA水或DFA磷酸缓冲液, DFA的载量为40 g/L. 所有的种子和发酵培养基均预先在121 °C灭菌20 min以防止DFA中微生物的污染. 培养中, 每4 h取样, 菌液中加入0.4%台酚蓝溶液, 在血细胞计数器中计算微生物细胞的死亡率, 并测定发酵液中β-葡萄糖苷酶的活性, 同时将微生物的形态与哈茨木霉CGMCC 2979纯培养物进行对比, 以保证无其它微生物污染. 培养96 h后, 加入12.5 mL乙醇, 混匀后室温静置12 h, 提取液过滤后进行HPLC分析.

为了获得最高收率的京尼平, 对影响转化率的影响因素(包括发酵时间, DFA浓度、发酵的温度和pH)进行了优化. 发酵时间优化范围为12–120 h, DFA载量40–120 g/L, 发酵温度设定为25, 30, 37 °C, pH值范围为2.1–7.1, 采用66.7 mmol/L磷酸缓冲液体系.

京尼平的转化率按照下面的公式计算:

$$\text{收率}(\text{mg/g}) = \frac{\text{转化后京尼平含量}(\text{mg})}{\text{转化前药材加入量}(\text{g})} \quad (1)$$

$$\text{转化率}(\%) = \frac{\text{转化后京尼平的含量}(\text{mol})}{\text{转化前京尼平苷的含量}(\text{mol})} \quad (2)$$

2.5. 从发酵液中分离纯化京尼平

收集发酵45 h的发酵液加入乙醇至终浓度为20%, 提取液在10000 × g下离心20 min, 除去菌体. 上清液命名为45-h样品. HPLC分析表明, 该样品含有2.4 mg/mL京尼平和0.9 mg/mL京尼平苷, 该样品进行XAD-16N树脂和硅胶层析.

XAD-16N树脂的处理方法为: 干树脂中加入95%乙醇中振荡24 h, 用去离子水充分洗涤备用. XAD-16N树脂的吸附条件由如下方法确定: 取八份10 mL 45-h样品, pH分别调至2.0–9.0后, 倒入50 mL三角瓶中. 向每个三角瓶中加入1 g处理好树脂, 25 °C下, 摇床中130 r/min条件下振荡12 h. 树脂吸附样品后, 弃去水相, 用去离子水洗涤树脂, 然后加入10 mL 80%乙醇水溶液, 在相同转速和温度下, 再振荡12 h. 乙醇洗脱液使用HPLC分析京尼平苷和京尼平含量, 以确定它们在树脂XAD-16N上的吸附量, 其计算公式如下:^[20]

$$q_e = (C_0 - C_e) \times V_i / ((1 - M) \times W) \quad (3)$$

C_0 和 C_e 分别是京尼平的初始和吸附平衡点的浓度(mg/mL), V_i 是溶液的体积(mL), M 是含水量(%), 按照文献[20]的方法确定本文中 M 值为65%, W 是树脂的重量(g).

采用动态吸附法考察乙醇浓度对京尼平吸附率的影响, 玻璃色谱柱(2.1 cm × 15 cm)装入45 g(湿重) XAD-16N树脂, 床体积为50 mL. 将100 mL 45-h样品调至pH 4.0后上柱, 吸附达到平衡后, 用3倍柱体积的去离子水洗涤, 然后用100 mL不同浓度的乙醇水溶液(30%, 40%, 50%, 60%, 70%, 80%, 90%和100%)阶梯梯度洗脱, 洗脱液进行HPLC分析.

硅胶柱层析在装有40 g硅胶的玻璃色谱柱(2.1 cm × 24 cm)中进行, 将30 mL 45-h样品减压浓缩成浸膏, 取50 mg浸膏上样, 硅胶柱用不同比例的氯仿-甲醇溶液(85:15, 84:16, 83:17, 82:18)洗脱, 洗脱液用HPLC分析.

在确定的XAD-16N树脂和硅胶层析条件的基础上, 转化后京尼平采用如下方法纯化: 取京尼平转化率最高(48 h)的样品200 mL, 用50 mL乙醇提取12 h后, 10000 × g离心20 min, 得到240 mL上清液. 上清液含有2.6 mg/mL京尼平和0.09 mg/mL京尼平苷(HPLC检测), 该样品命名为48-h样品. 该样品调pH至4.0后, 上样至装有145 g XAD-16N的色谱柱中(3.0 cm × 30 cm), 色谱柱首先用2倍体积30%乙醇水溶液(pH 4.0)洗涤, 再用2倍体积60%乙醇水溶液(pH 6.5)洗脱, 洗脱液用UV检测器检测收集, 减压浓缩成浸膏. 在色谱柱(4.0 cm × 25 cm)中装入150 g硅胶, 将浓缩的浸膏用少量甲醇溶解后上样, 色谱柱用600 mL氯仿-甲醇(83:17)洗脱. 含有京尼平的馏分浓缩至干, 用2.0 mL甲醇水溶液(66.7%)洗涤后, 得到纯的京尼平.

2.6. 纯化β-葡萄糖苷酶

在最优条件下获得的发酵液, 10000 × g离心10 min去除菌体. 缓慢加入固体硫酸铵至75%饱和度, 于4 °C放置过夜, 10000 × g离心10 min, 收集蛋白沉淀. 粗蛋白溶于66.7 mmol/L磷酸钠缓冲液(pH 5.0)去除不溶物(10000 × g, 10 min). 上清液先后用0.45 μm膜过滤和超滤浓缩. 样品上样至DEAE Sepharose色谱柱(3.0 cm × 12 cm), 该柱预先用上述磷酸钠缓冲液平衡. 收集未结合馏分, 超滤浓缩后, 上样至Superdex 200柱(3.0 cm × 21 cm), 该柱也预先用磷酸钠缓冲液平衡, 用相同缓冲液洗脱, 流速为1 mL/min. 色谱过程中采用280 nm检测蛋白, 所有馏分均进行β-葡萄糖苷酶活性分析.

2.7. 酶学特性

酶学特性主要考察了温度(20–70 °C), pH(2.0–7.0, 66.7 mmol/L磷酸钠缓冲液)对酶活的影响. 同时进行了500–800 nm的扫描考察副产物(蓝色素)的生成.

底物特异性实验选用栀子中京尼平苷和盾叶薯蓣

中甙体皂苷, 包括盾叶新苷、三角叶皂苷、薯蓣皂苷元-三葡萄糖苷、薯蓣皂苷元-二糖苷、延龄草次苷. 底物溶液是将1 mg京尼平苷或甙体皂苷溶于1 mL磷酸钠缓冲液, 加入0.2 mL纯酶液, 对于甙体皂苷, 反应在50 °C进行24 h, 加入0.2 mL正丁醇, 涡旋1 min, 底物和产物被提取至丁醇相进行HPLC检测. 对京尼平苷, 反应也在50 °C下进行, 反应时间为1.0, 1.5, 2.0, 2.5和3.0 h, 水中加热5 min终止反应.

纯化的京尼平苷-β-葡萄糖苷酶的 K_m 和 V_{max} 值, 用Lineweaver-Burk曲线获得, 测定条件为50 °C, pH 5.0, 底物京尼平苷浓度为1.18–2.95 mmol/L.

3. 结果与讨论

3.1. DFA中京尼平苷含量的确定以及乙醇浓度对京尼平苷的影响.

索氏提取实验显示, 京尼平苷在DFA中的含量是 64.1 ± 1.5 mg/g, 溶剂提取后, 京尼平苷浓度与该值比较以确定提取率. 为了确定京尼平苷和京尼平提取物均有效的方法, 进行了乙醇对京尼平苷和京尼平收率影响的研究. 研究表明, 京尼平苷主要以固体形式存在于栀子果实中, 而生物转化后会被分泌到上清中^[16], 所以我们选用DFA和京尼平的标准品作为提取对象. 使用0%, 20%, 50%, 70%和100%乙醇水溶液提取DFA中京尼平苷的收率分别为 55.4 ± 0.8 , 63.8 ± 2.4 , 61.9 ± 2.9 , 51.6 ± 0.5 和 26.8 ± 1.3 mg/g DFA. 使用20%乙醇提取时收率最高, 与索氏提取法相同(提取率为99.5%). 用20%乙醇溶液提取4 mg/mL的京尼平标准品, 京尼平的收率达 $99.4 \pm 0.2\%$. 因此, 生物转化前后的样品均加入乙醇至终浓度为20%.

3.2. 哈茨木霉CGMCC 2979生物转化栀子中京尼平苷

哈茨木霉CGMCC 2979接入含DFA粉末的培养基中, 于30 °C培养96 h后, 京尼平苷和京尼平浓度分析显示, 93%的京尼平苷转化为京尼平. 这表明哈茨木霉CGMCC 2979可以直接转化含有DFA的栀子, 无需酶的纯化或者从药材中提取京尼平苷的步骤. 在发酵过程中, 哈茨木霉分泌的β-葡萄糖苷酶可以水解栀子果实中的京尼平苷, 副产物为葡萄糖. 而后者可作为真菌的碳源, 从而减少了由葡萄糖积累导致的负反馈, 使得生物转化的效率提高.

为了获得更高的收率并缩短发酵时间, 进行了发酵条件的优化. 培养温度影响实验结果显示, 25, 30和37 °C时转化率分别为 $92.6\% \pm 3.7\%$, $97.7\% \pm 0.9\%$ 和 $88.6 \pm$

2.2%, 可见, 30 °C 为获得京尼平的最佳温度, 该温度也最适合哈茨木霉的生长^[15,21], 京尼平的产生和微生物生长之间关系密切。

DFA的载量对转化率影响也较大(图1(a)), 京尼平苷载量为40到80 g/L时, 京尼平的转化率可以超过95%, 如果超过80 g/L, 转化率则会大幅度降低。

另一个重要影响因素是培养基的pH值(图1(b))。当培养基的pH = 2.1–5.1时, 转化率低于60%; 至6.1时, 转化率增加到了98.3% ± 3.1%; 继续提高pH至7.1, 转化率将降低。可见, pH = 6.1最适于微生物产生转化京尼平苷为京尼平的 β -葡萄糖苷酶, 同时pH 6.1磷酸缓冲液不仅能够稳定培养基的pH值, 还能为微生物提供磷源, 促进微生物的生长和酶的分泌。

经优化, 确定最佳培养条件为DFA载量80 g/L, 30 °C 以及pH 6.1。该培养条件培养基配方简单, 主要利用药材中有效成分, 不需要额外补充碳源和氮源, 整个转化过程简便、高效。上述优化培养条件的时间进程实验表明, 京尼平的转化率在24到48 h快速提高, 同时生物转化前京尼平苷的含量为13.2 mmol/L, 在生物转化初期, 随着发酵进行缓慢降低, 40 h以后急速下降。在48 h京尼平的含量达到最高值12.9 mmol/L (3.0 g/L), 是*Penicillium nigricans*转化获得京尼平的1.88倍(6.88 mmol/L, 1.6 g/L), 转化率和收率分别为97.8%和3.6 mg/g DFA (图2)。发酵时间文献报道的缩短了55.6%^[14]; 超过了48 h, 转化率逐渐降低(50 h为91.4% ± 1.3%, 52 h为85.5% ± 4.8%, 55 h为83.6% ± 3.3%)。

台酚蓝染色实验表明发酵最初的32 h, 细胞的死亡率低于10%, 40和48 h分别增加到13.0%和16.6%, 但发酵终止时(55 h)细胞死亡率提高到了25.0%。时间进程中 β -糖苷酶活性显示, β -糖苷酶活性最初是在12 h被检测出, 然后在40 h达到最佳值(23.77 U/mL), 55 h下降到19.76 U/mL (图2)。发酵液的菌体形态观察显示, 发酵液的菌体和哈茨木霉CGMCC 2979的形态一致。结果表明, 转化京尼平苷为京尼平的 β -葡萄糖苷酶确是由哈茨木霉CGMCC 2979产生, 而40 h酶活下降可能与部分真菌细胞死亡有关。

3.3. 从发酵液中分离纯化京尼平

静态吸附实验(图3(a))表明京尼平可以被XAD-16N吸附, 吸附能力受初始pH影响。当初始pH从2.0增加到4.0过程中, XAD-16N对京尼平的吸附量 q_e 逐渐增加, 进一步增加pH值, 吸附量略有降低; 至7.0, 洗脱液中未能检测到京尼平, 表明此时京尼平不能被吸附到XAD-16N

上, 所以pH = 4.0最适于XAD-16N吸附京尼平。而在pH = 2.0–7.0范围内, XAD-16N对京尼平苷的吸附量一直保持在约3.2 mg/g树脂。

从XAD-16N树脂柱上洗脱京尼平, 可以通过阶段提高乙醇浓度梯度的方式实现。动态吸附曲线(图3(b))表明, 洗脱液中京尼平浓度随着洗脱剂中乙醇的含量增加而增加, 至50%时, 达到最大值; 继续增加乙醇浓度, 洗脱液中京尼平的浓度开始下降。为了获得最大的京尼平纯化收率和相对高的除杂作用, 洗脱条件为: 色谱柱首先用30%乙醇水溶液洗脱除去京尼平苷, 然后用60%乙醇水溶液洗脱获得京尼平。京尼平和京尼平苷的收率分别为92.2%和32.7%。结果表明, 当上样条件为20%乙醇水溶液(pH 4.0)时, 绝大部分的京尼平苷不能被XAD-16N吸附, 或者被30%乙醇水溶液(pH 4.0)的溶液在洗涤时除去。

因为发酵液中成分比较复杂, 为了获得高纯度京尼平, 硅胶柱层析也被用于京尼平和京尼平苷的分离纯化。结果显示, 吸附到硅胶柱上的京尼平苷可以被82:18 (v/v)甲醇-氯仿洗脱除去, 而京尼平则主要在83:17 (v/v)甲醇-氯仿馏分中, 所以83:17 (v/v)的甲醇-氯仿被用作京尼平的洗脱条件。

接着, 将上述得到的最优条件用于纯化48 h的发酵液, 此时京尼平苷转化为京尼平的收率最高。采用XAD-16N树脂和硅胶层析偶联的分离、纯化方式, 可以有效地去除未转化的京尼平苷、色素和发酵液中其它杂质, 从200 mL发酵液中共得到389.0 mg无色的京尼平, HPLC分析显示其纯度为98.3%。XAD-16N和硅胶层析的收率分别为87.1%和77.0%, 纯化的总收率为62.3%。

质谱分析显示, 纯化的产物在负离子模式下有两个离子峰, 225和261分别代表[M-H]⁻和[M+Cl]⁻, 所以分离得到的化合物分子量为226, 与京尼平的一致。其核磁共振的数据如下: ¹H NMR(CH₃OD) δ : 7.53(s, H-3), 5.83(s, H-7), 4.80(d, J = 8.4 Hz, H-1), 4.32(d, J = 14.4 Hz, H-10a), 4.22(d, J = 14.4 Hz, H-10b), 3.70(s, -OCH₃), 3.16(m, H-5), 2.86(1H, m, H-6b), 2.50(1H, m, H-9), 2.06(1H, m, H-6a)。¹³C NMR(400 MHz, CH₃OD) δ : 170.0(-CO₂-), 154.4(C-3), 145.7(C-8), 128.4(C-7), 111.9(C-4), 97.9(C-1), 61.8(C-10), 51.8(-OCH₃), 48.6(C-9), 40.1(C-6), 37.8(C-5), 与文献[4]报道一致。

据我们所知, 在用微生物直接转化药材的研究中, 哈茨木霉CGMCC 2979转化栀子京尼平苷的发酵时间最短, 转化率以及得到的京尼平的浓度均最高, 此外采

用XAD-16N和硅胶还能够很好地选择性分离京尼平和京尼平苷, 构成了一种高效获得高纯度京尼平的方法。

3.4. 京尼平苷- β -葡萄糖苷酶的纯化和酶学性质

为了研究酶学性质, 首先采用了硫酸铵沉淀、DEAE sepharose和Superdex 200等分离手段对哈茨木霉中京尼平苷- β -葡萄糖苷酶进行了纯化。DEAE Sepharose未结合馏分显示出 β -葡萄糖苷酶活性, 该馏分经过Superdex柱层析后获得纯酶, 纯化倍数为16倍, 收率为14.7%。使用pNPG做为底物进行了底物特异性研究, 测定纯化的京尼平苷- β -葡萄糖苷酶的活性为72.0 U/mg, 酶纯化的数据见表1。

SDS-PAGE(图4)显示, 纯化京尼平苷- β -葡萄糖苷酶的分子量约为76.5 kDa, MALDI/TOF MS分析显示其准确分子量为74.4 kDa, 小于从小肠厌氧菌和黑曲霉Au0847中分离的京尼平苷- β -葡萄糖苷酶的90和230 kDa^[9,22]。

哈茨木霉中分离得到的京尼平苷- β -葡萄糖苷酶在pH 4.0–5.0显示最大酶活, pH 6.0时转化率略有降低, 但依然达到90%以上, pH达到7.0时, 转化率降至70%。该酶的活性随着温度的升高而升高, 在50 °C达到最高, 60 °C后显著降低, 70 °C失去酶活。时间进程显示, 京尼平主要在发酵40 h时产生, 同时也表现出最大 β -葡萄糖苷酶酶活。可见0–40 h是产酶的阶段, 40–48 h是生物转化的阶段。在生物转化阶段, 提高发酵温度将缩短生物转化时间、提高产物收率^[14]。结合该酶的最适温度40–50 °C, 可认为在转化阶段(40–48 h)可将温度提高到40–50 °C, 从而会进一步缩短转化时间, 提高转化效率。

该酶在50 °C, pH 5.0的条件下, 经1和2 h可以将91.0%和98.9%的京尼平苷转化为京尼平。 K_m 和 V_{max} 值

分别为3.6 mmol/L和775 μ mol/h mg protein ($y = 0.00467x + 0.00129$, $R^2 = 0.9746$), 该酶的 K_m 值和从黑曲霉Au0847中分离得到的酶的相近(2.93 mmol/L)^[9]。该酶不能够水解三角叶皂苷和盾叶新苷的 α (1 $\rightarrow$ 2)鼠李糖苷键, 以及不能水解含有 β (1 $\rightarrow$ 3)和 β (1 $\rightarrow$ 4)糖苷键的甾体皂苷, 如盾叶新苷, 三角叶皂苷、薯蓣皂苷元-三葡萄糖苷、薯蓣皂苷元-二葡萄糖苷; 同时也不能水解延龄草次苷, 该化合物也含有单一的 β -1-4葡萄糖苷键, 但其苷元京尼平苷的苷元结构不同。由此可见, 从哈茨木霉中纯化的酶是一种特异性的京尼平苷- β -葡萄糖苷酶, 可以特异性识别京尼平苷的苷元。一种分子量为75 kDa的 β -葡萄糖苷曾经从哈茨木霉中分离出, 底物特异性研究表明, 该酶可以以一些烷基糖苷、二糖以及寡糖作为底物。但它以天然产物为底物的酶学研究未见报道^[23]。

扫描500–800 nm范围内吸收光谱发现, 京尼平苷- β -葡萄糖苷酶转化京尼平苷前后的吸收光谱未发生明显变化, 表明纯化的京尼平苷- β -葡萄糖苷酶不与京尼平反应。表现出与普通商业化酶不同的特性, 这可能是该酶在直接转化药材中京尼平苷时转化率较高的原因之一。

4. 结论

我们发现了一种简便、高效制备京尼平的方法, 它采用哈茨木霉CGMCC 2979转化梔子中的京尼平苷, 生成的京尼平从发酵液中使用XAD-16N和硅胶柱层析分离, 纯化收率62.3%, 纯度98.3%。和以前的同类工作相比, 该法转化时间最短、收率和京尼平的浓度最高。另外, 从哈茨木霉中分离得到的京尼平苷- β -葡萄糖苷酶对京尼平苷显示较高的特异性水解的活性。该方法具有替代现有京尼平制备方法的潜力。