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Purification and Characterization of β-*D*-glucosidase Hydrolyzing Swertiamarin into Erythrocentaurin and 5-ethylidene-8-hydroxy-3, 4, 5, 6, 7, 8-hexahydro-1H-pyrano [3, 4-C]pyridine-1-one from *Aspergillus niger* CICC 2169

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The β-*D*-glucosidase involved in the biotransformation of swertiamarin to 5-ethylidene-8-hydroxy-3,4,5,6,7,8-hexahydro-1H-pyrano[3, 4-c] pyridine-1-one and erythrocentaurin was purified and characterized. The enzyme had a molecular weight of 88 kDa as determined by SDS polyacrylamide gel electrophoresis. The purified enzyme was optimally active at 55-60°C and pH 5.0. The enzyme was stable at pH 5-7 and 60°C, and specified on β-(1-4)-glucosidic linkage. The affinity constants for ρ-NPG and swertiamarin were 0.58 and 0.26 (mM),respectively. So the catalytic constants for ρ-NPG and swertiamarin were 186.8 and 79.6 (μmol min^{-1} mg^{-1}), respectively. Ca^{2+}, Cu^{2+}, Zn^{2+}, and Fe^{3+} (0∼120 mM) inhibited the β-*D*-glucosidase, while Mg^{2+} and Mn^{2+} (each 0∼120 mM) activated this enzyme. Glucose had a high inhibitory effect on the enzyme and the inhibition constant was 0.13 and thus the concentration of glucose in the broth for producing the β-*D*-glucosidase should be strictly controlled no more than 8mg/l. The metabolic pathway and the role of the purified β-*D*-glucosidase were fully discussed.

Key words: Erythrocentaurin, 5-ethylidene-8-hydroxy-3, 4, 5, 6, 7, 8-hexahydro-1H-pyrano [3, 4-c] pyridine-1-one, Purification, ^{2}-*D*-glucosidase, Swertiamarin

Swertiamarin is one of major physiologically active ingredients of *Zangyinchen*, an important Chinese traditional herb drug with hepatoprotective effect[1]. Previous studies showed that the swertiamarin can be metabolized by *Aspergillus niger* CICC 2169 into two metabolites, 5-ethylidene-8-hydroxy-3, 4, 5, 6, 7, 8-hexahydro-1*H*-pyrano [3, 4-*c*]-pyridine-1-one (EHP) and erythrocentaurin. The former is a novel alkaloid with anti-inflammatory activity[2] and the latter a known active compound. The β-*D*-glucosidase was found to be the rate-limiting enzyme of the metabolic pathway. To optimize the biotransformation condition, the β-*D*-glucosidase from *Aspergillus niger* CICC 2169 was purified and characterized.

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RESULTS AND DISCUSSION

Enzyme production

The result of enzyme fermentation by *Aspergillus niger* (Table 1) indicated that the cell growth reached maximum after 72h of fermentation and the maximum enzyme production was obtained

Table 1. The time course of growth of *A. niger* and the production of β-*D*-glucosidase*

	Fermentation time (h)									
	0	12	24	36	48	60	72	84	96	108
Cell weight (mg/ml)	0	0.2	1.3	5.2	16.9	18.7	21.3	20.6	19.7	21.5
Enzyme activity (U/ml)	0	0.2	1.6	4.7	15.9	28.4	39.5	48.1	58.3	47.6

*The time course was studied with 5 l fermentator containing 2.5 l medium

at 96h. The fermentation was, therefore, carried out for 96h.

Enzyme purification and Enzyme molecular weight

When cultured with 75% $(NH_4)_2SO_4$, most of the β-*D*-glucosidase were precipitated. The peak eluted by 180mM KCl displayed activity of catalyzing swertiamarin into erythrocentaurin and EHP (Fig1), and formed one band on SDS-polyacrylamide gel electrophoresis (Fig2), which suggested a pure protein. The pure enzyme activity (yield) was 8.3% of total enzyme obtained from the purification (Table 2). The specific activity of the β-*D*-glucosidase was 156.2 U/mg, an increase of 13.5 times. The molecular weight of the β-*D*-glucosidase was 88kDa (Fig3).

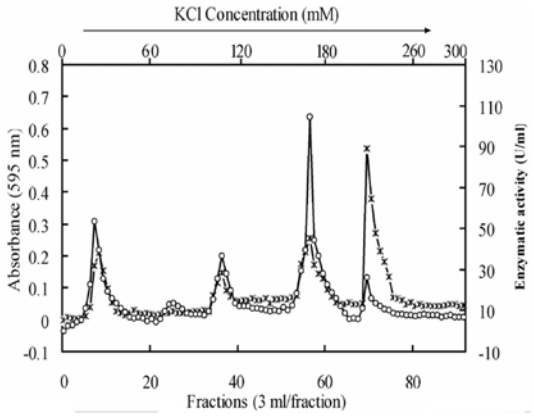

Fig. 1.

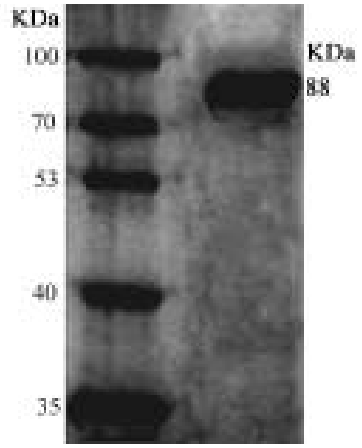

Fig. 2.

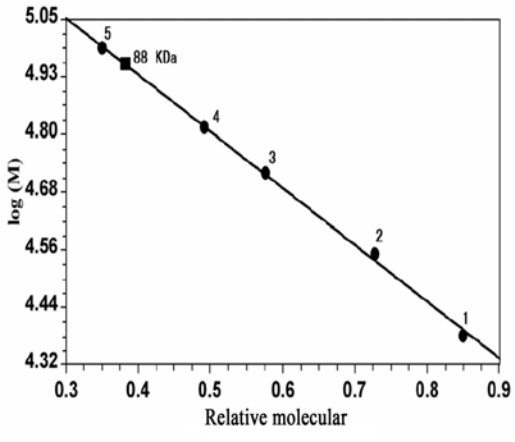

Fig. 3.

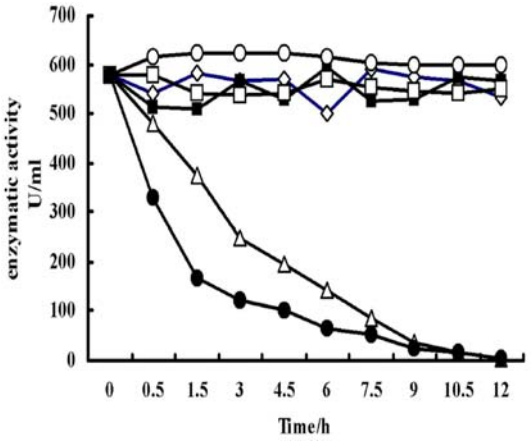

Fig. 4.

Table 2. β-*D*-glucosidase purification

Step	Volume (ml)	Total protein (mg)	Specific activity (U/ml)	Yield (%)
Fermentation	2500	5381	26.2	100
precipitation	250	3635	32.8	69.5
DEAE-sepharse	70	28.7	156.2	8.3

Table 3. Effect of metallic ions on β-*D*-glucosidase (relative activity, %)

Concentration	Relative activity (%)					
(mM)	$CaCl_2$	$MgSO_4$	$FeCl_3$	$ZnCl_2$	$CuSO_4$	$MnSO_4$
0[a]	100	100	100	100	100	100
2	98.5	103.8	97.1	96.4	94.6	102.1
5	93.7	109.3	88.6	95.9	90.1	103.3
10	85.4	113.8	83.2	86.7	80.2	110.2
50	59.2	120.5	69.1	33.9	21.8	115.5
80	13.1	127.9	34.2	17.5	ND[b]	121.7
100	ND	138.3	15.2	ND	ND	131.3
120	ND	133.6	ND	ND	ND	127.9

[a] The concentration of the Ca^{2+}, Mg^{2+}, Fe^{3+}, Zn^{2+}, Cu^{2+} and Mn^{2+} were 5.4, 12.3, 8.6, 18.1, 7.3 and 11.8 mg/l, respectively.
[b] ND, Not determined.

Properties of β-D-glucosidase

The result of estimating the temperature dependence showed that the optimal temperature for maximal activity ranged from 50-60°C when preparations were incubated for 2h in 100mM phosphate buffer (pH 5.0). Under the optimal temperature, the purified enzyme was proved to have an optimal pH 5.0.The data of thermostability and pH stability (Fig4 and Fig5) suggested that the enzyme was highly stable at temperature up to 60°C. When incubated at 70 and 80°C, the activity of β-*D*-glucosidase decreased dramatically and the residual activities were 5.93 and 4.55 U/mg, respectively at 12h. The enzyme was fairly stable at pH 5.0, 6.0 and 7.0, and retained full activity after incubated for 12h at 50°C. The metallic ions have great effects on the enzyme (Table 3). The β-*D*-glucosidase was inhibited by Ca^{2+}, Zn^{2+}, Fe^{3+}

Table 4. Effect of the β-*D*-glucosidase on different substrates

Substrate	Configuration of glycoside linkage	Relative initialrate of hydrolysis (%)
cellobiose	(1→4) βGlc	98
Lactose	(1→4) βGal	ND
maltose	(1→4) αGlc	ND
laminarin	(1→3) βGlc	88
ρ-NPG	(1→4) βGlc	100
swertiamarin	(1→4) βGlc	98

ND, Not determined

Table 5. Affinity and catalytic constants calculated for β-D-glucosidase

	β-D-glucosidase	
	K_m (mM)	K_{cat} (μmol min^{-1} mg^{-1})
ρ-NPG	0.58	186.8
Swertiamarin	0.26	79.6

and Cu^{2+}, giving 59.2, 69.1, 33.9 and 21.8 % of activity of control at concentration of 50mM. The β-*D*-glucosidase was activated by Mg^{2+} and Mn^{2+}. Substrate specificity of the enzyme was determined using different polymers, containing either β-1,4 and β-1,3 linkages. The purified enzyme showed high activity towards cellobiose and ρ-NPG, but decreased quantity of reducing sugar (88% relative to that of cellobiose and ρ-NPG) was liberated from swertiamarin (Table 4). In the reaction system containing lactoseandmaltose and laminarin, no β-glucosidase activity were determined.

Kinetic constants of β-D-glucosidase on ρ-NPG and swertiamarin

The affinity constants (K_m) calculated for ρ-NPG and swertiamarin were 0.26 and 0.58 mM, respectively. The catalytic constants (K_{cat}) for *Á*-NPG and swertiamarin were 186.8 and 79.6 µmol min^{-1} mg^{-1}, respectively. The decreased degradation of swertiamarin would be due to its lower K_{cat} value (Table 5).

Inhibition constants of glucose on β-D-glucosidase

Glucose was found to be a strong competitive inhibitor of the purified enzyme with the inhibition constant of 0.13.

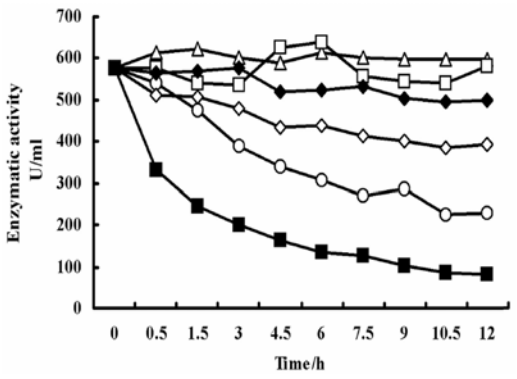

Fig. 5.

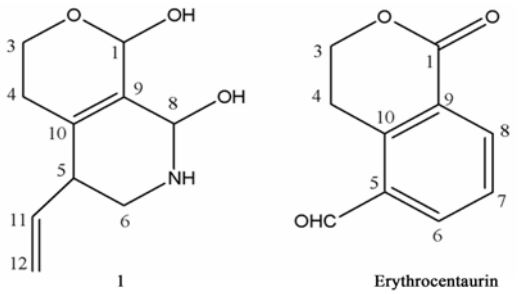

Fig. 6.

DISCUSSION

β-*D*-glucosidase is responsible for hydrolysis of glycoconjugated precursors[6] and the rate-limiting enzyme involving the biotransformation of swertiamarin to EHP and erythrocentaurin, two active compounds. To optimize the production of metabolites, the extracellular β-*D*-glucosidase from *Aspergillus niger* was isolated, purified and characterized. The molecular weight was determined as 88 kDa by SDS-polacrylaminde gel electrophresis, which is smaller than the molecular weight of β-*D*-glucosidase from *Achatina fulica* (110 kDa)[7], but larger than that of β-*D*-glucosidase from *Tarocco* cultivar (65 kDa)[8]. It is known that β-*D*-glucosidases play an important role in the solubilization and reconstitution of biological membranes[9]. It was therefore not surprising to observe β-*D*-glucosidase production continued to increase after 24h of the cell growth reaching its maximum production. The overall purification procedure resulted in recovery of 8.3% and 152.6-fold increase in specific activity. This recovery was larger than report[10], which indicated an effective purification procedure[11]. The enzyme was optimally active at 55-60 °C and pH 5.0, which is similar to most of β-*D*-glucosidase. The enzyme was stable at pH 5 to 7, and at temperature up to 60°C which was in accordance with fungal β-*D*-glucosidase[12] and piceid-β-*D*-glucosidase[13], but different from the report of Villena[14] where the optimal activity condition was pH 4-5 at 40°C. The decrease in activity under pH values lower than 5.0 or higher than 7.0 may be due to the isoellectric point of the β-*D*-glucosidase[15]. Under the optimum conditions the specificity activity was 615.27 U/mg on ρ-NPG, which is similar to most of β-*D*-glucosidases from fungi[16]. β-glucosidases may be divided into three groups on the basis of their substrate specificity: aryl-β-glucosidases, cellobiases and β-glucosidases[17]. The specificity of the β-*D*-glucosidase on β-(1-4)-glucosidic linkage indicated that the enzyme may be included in the third group. Glucose is a competitive inhibitor of β-glucosidase and the inhibition constant of glucose was 0.13, which is much lower than the report[18]. It means that glucose had a greater inhibitory effect on the β-glucosidase. Thus the concentration of glucosidase in the optimal culture should be lesser

than general culture which contains 20g/l glucosidase.

Mg^{2+} and Mn^{2+} are capable of activating the β-*D*-glucosidase. It meant that the optimal medium for biotransformation should contain 100mM Mg^{2+} and 100mM Mn^{2+}. Ca^{2+}, Zn^{2+}, Fe^{3+} and Cu^{2+} have great inhibitory effects on the enzyme. In tract quantities these inhibitory metals, however, would server as essential factors in the growth of *Aspergillus niger*. Thus Ca^{2+}, Zn^{2+}, Fe^{3+} and Cu^{2+} should not be additionally added into the optimal medium and the essential concentrations of these ions would be contained in other ingredients such as peptone and yeast extracts.

Swertiamarin was firstly hydrolyzed by β-*D*-glucosidase into corresponding aglycone (1) (Fig 6). The aglycone was then transformed into 2, which was then catalyzed by CYP450 responsible for epoxidation reactions. Under the catalysis of CYP450s, 2 was finally metabolized into erythrocentaurin. EHP was directly produced from aglycone, and the catalysis mechanism, however, were still unknown. When cultured with medium without *A.niger*, swertiamarin was stable at 37°C for 6 days. Furthermore, we failed to determine and separate the aglycone from the biotransformation culture, which suggested that the β-*D*-glucosidase is the rate-limiting enzyme of the whole metabolismpathway.

The further study of optimal fermentation conditions and the scaled production of EHP and erythrocentaurin should be central on how to improve the catalytic properties of the β-*D*-glucosidase.

MATERIALS AND METHODS

Standard swertiamarin (over 99% in purity) was purchased from Tianjin Institute for Drug Control (Tianjin, China). Swertiamarin used for biotransformation was extracted and purified from *Swertia mussotii* Franch and approximately 96% pure. Methanol and acetonitrile were supplied by Aldrich Chemical Company (Gillingham, UK). ρ-nitrophenyl-β-*D*-glucoside (ρ-NPG) and ρ-nitrophenyl (ρ-NP) were obtained from AppliChem (Gatersleben, Germany).

Enzyme production

Fermentation was performed in a 5 l fermentor with 2.5 l working volume at 28°C and mixing at 350 rpm. The medium consisted of glucose8g/l, peptone5g/l, yeast extract5g/l, $KH_2PO_4$5g/l, NaCl 5g/l, $MgSO_4 \cdot 7H_2O$ 1g/l, $MnSO_4 \cdot 4H_2O$ 1g/l and pH 6.0. The pH was adjusted during the fermentation with 10% NaOH to 6.0. The fermentor was aerated at 18 l/min. Enzyme production continued for 5 days. After terminating the fermentation process, the broth was filtered through a glass filter, freeze-dried and stored as crude enzyme at 4°C for further purified.

Purification of β-D-glucosidase

Pellets of $(NH_4)_2SO_4$ were slowly added to the crude enzyme solution with shaking to 75% saturation and stored at 4°C overnight. The mixture was centrifuged to collect the protein precipitate. This crude protein was re-dissolved in distilled water and dialyzed against 20mM acetate buffer (pH 5.0). After removing the non-dissolved fraction by centrifugation, the enzyme solution was fractionated on a column (Φ 1.5×10 cm) of DEAE-Sepharose. The column was then eluted by a KCl gradient from 0 to 300mM in 20mM Tris–HCl buffer (pH5.5) to collect the enzyme fractions. Fractions giving β-*D*-glucosidase activity were combined and freeze-dried.

Enzyme analysis

The β-*D*-glucosidase activity was determined as follows: a 0.1 ml sample was mixed with 1ml of 1mM ρ-NPG and kept at 55°C for 30min. The reaction was stopped by adding 2ml of 0.5mM Na_2CO_3. The absorbance was measured at 400nm by spectrophotometer. 0.1ml of water instead of enzyme solution was used as the blank. One unit of activity was represented as 1nmol min^{-1} of ρ-NP. The assay was done in triplicate on each sample and the mean of the obtained values was used.

Protein assay

Protein content was determined according to Bradford[3] at 595nm using correspondingly diluted phase without sample as blank. Bovine serum albumin was used as a standard. The determination was done in triplicate on each sample and the mean was used.

Determination of properties

The molecular weight of purified enzyme were determined by SDS-polyacrylamide gel electrophoresis using trypsin (20.1 kDa), carbonic anhydrase (30 kDa), ovalbumin (45 kDa), bovine serum albumin (66 kDa) and phophorylase b (97

kDa) as standard proteins. The temperature optimum of β-*D*-glucosidase activity was assayed in the temperature range from 20 to 80°Cin 100 mM, pH 5.0 phosphate buffer.

The optimal pH for β-*D*-glucosidase activity was tested in the pH range from 2.5 to 8.0 in 100 mM phosphate buffer at 50°C. The stability under different temperatures was assayed as follows: enzyme solutions were incubated at 30, 40, 50, 60, 70 and 80°Cfor 12h in 100mM, pH 5.0 phosphate buffers. Samples (each 0.1ml) were taken at 0, 1.5, 3.0, 4.5, 6.0, 7.5, 9.0, 10.5 and 12.0h and the activity of enzyme was assayed by the standard assay method.

The pH stability of *β-D*-glucosidase was tested as follows: the enzyme was dissolved in phosphate buffer with pH values of 3.0, 4.0, 5.0, 6.0, 7.0 and 8.0, respectively. Samples (each 0.1ml) were taken at 0, 1.5, 3.0, 4.5, 6.0, 7.5, 9.0, 10.5 and 12.0h and the activities of enzyme were determined with the standard assay method.

The inhibitory effect of glucose on the β-*D*-glucosidase was measured with ρ-NPG as substrate [4]. Inhibition constant (K_i) was determined at two concentrations using Michaelis-Menten equation.

The effects of Ca^{2+}, Mg^{2+}, Fe^{3+}, Zn^{2+}, Cu^{2+} and Mn^{2+} on the β-*D*-glucosidase were tested at concentrations of 0, 2, 5, 10, 50, 80, 100 and 120mM.

Substrate specificity

To investigate the ability of the enzyme hydrolyzing β-glucosidic links of specificity substrates, the β-*D*-glucosidase activity was assayed by measuring the amount of glucose released from substrates at the optimal temperature and pH. Substrate specificity of the enzyme was determined using two different concentrations (1 and 10mM) of cellobiose, lactose, maltose, laminarin, *Á*-NPG and swertiamarin, respectively.

Kinetic parameters

Kinetic constants were determined on *Á*-NPG and swertiamarin. Affinity (K_m, mM) and catalytic (k_{cat}, μmol min^{-1} mg^{-1}) constants were obtained using linear regression plots of the Michaelis-Menten equation of Lineweaver-Burk.

Determination, isolation and structure of metabolites

Swertiamarin and its metabolites were first determined in broth of *A.niger* CICC 2169 according to our previous method[5]. The metabolites were then isolated and elucidated in previous reported methods[2].

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