PRODUCTION OF ACTEOSIDE FROM *CISTANCHE TUBULOSA* BY β-GLUCOSIDASE

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ABSTRACT

Acteoside and echinacoside are the major active components of Herba Cistanches. Facilitated β-glucosidation was investigated as a means of increasing harvest of acteoside from *Cistanche tubulosa*. Fresh *Cistanche tubulosa* was treated by microwave moisture processing to inactivate enzymes. β-Glucosidase is capable of hydrolyzing echinacoside for the production of acteoside, so six β-glucosidases were compared for their efficiency, specific activities and kinetic parameters for conversion to acteoside. The acteoside and echinacoside content was found to be higher after microwave processing than by other previously reported methods. The results showed that β-glucosidase isolated from microorganisms (*Trichoderma* sp.) had highly specific activity towards echinacoside, and there was a 4.83 fold increase in the concentration of acteoside after an incubation period of 2 h. This is the first report of the potential application of β-glucosidases for the facilitated conversion of echinacoside to acteoside in Herba Cistanches extract.

Keywords: *Cistanche tubulosa*, acteoside, echinacoside, microwave, β-glucosidase.

INTRODUCTION

Acteoside (verbascoside) and echinacoside are members of the phenylethanoid (arylethyl) glycosides, a naturally occurring water-soluble group of polyphenolic compounds with many pharmacological properties. Acteoside in particular has been shown to protect rat cortical cells in primary culture from the neurotoxicity that would otherwise be induced by glutamate (Koo *et al.*, 2006). In pharmacokinetics studies, acteoside could be detected, by a sensitive liquid chromatography-tandem mass spectrometry (LC-MS/MS) method, in brain tissue after intravenous administration. This finding supports its sedative effect on the central nervous system (Wu *et al*., 2006). Acteoside also selectively suppressed activation of the transcription factor activating protein (AP)-1, which may be essential for nitric oxide synthase induction in lipopolysaccharide (LPS)-treated macrophages (Lee *et al*., 2005). In addition, acteoside at lower doses had a positive effect on respiratory burst (an anti-microbial response) of neutrophils (Akbay *et al.*, 2002), and a suppressive effect on lung metastasis of B16 melanoma cells (Ohno *et al.*, 2002). Echinacoside also has demonstrated pharmacological actions including hepatoprotective effects (Wu *et al.*, 2007) and antioxidant activities (Pellati *et al.*, 2004). It was reported that acteoside (IC_{50} = 4.6 μM) and echinacoside (10.2 μM) inhibited D-GalN-induced death of hepatocytes, and acteoside (17.8 μM) and echinacoside (31.1 μM) also reduced TNF-α-induced cytotoxicity in L929 cells (Morikawa *et al.*, 2010). The hepatoprotective activity of acteoside was greater than that of echinacoside, presuming that the 6'-O-β-D-glucopyranosyl moiety in the structure of echinacoside may reduce the activity. However, it remains to be determined which phenylethanoid glycoside (PhG) has the greater biochemical activities in this herb extract, and which structural feature results in the diverse biological and pharmacological properties of PhGs.

Acteoside is widely distributed in many medicinal plants, including *Ligustrum purpurascens* (Wong *et al.*, 2001), *Verbascum densiflorum*, *V. phlomoides* (Klimek *et al.*, 2010), and *Kigelia africana* (Santoro *et al.*, 2008). But the amount of acteoside in these plants is very small (for example, less than 2.1% of dry weight) (Klimek *et al.*, 2010; Santoro *et al.*, 2008; Wong *et al.*, 2001). *Cistanche tubulosa* (Schenk) R. Wight, one of the *Cistanche* (Orobanchaceae) species known as Herba Cistanches in China, has higher contents of acteoside and echinacoside than other medicinal plants (Cai *et al.*, 2007; Klimek *et al.*, 2010; Santoro *et al.*, 2008; Shi *et al.*, 2009; Wong *et al.*, 2001). Herba Cistanches (Rou Cong Rong), referring specifically to the dried succulent stems of *Cistanche* spp., is considered a superior tonic and has earned the honorific "Ginseng of the deserts". Herba Cistanches is used medicinally in the treatment of kidney deficiency, impotence, female infertility and senile constipation (Jiang and Tu, 2009).

In 2001, Lei *et al.* first reported the metabolic pathway of PhGs in the gastrointestinal tract of beagle dogs, where a portion of echinacoside is transformed into acteoside by intestinal bacteria (Lei *et al.*, 2001). Thus it was found that acteoside, an active ingredient of *Cistanche* spp., can be obtained by deglycosylation of the intermediate echinacoside. Deglycosylation of glycosides can be performed by using chemical methods such as acid hydrolysis, alkaline hydrolysis, and heat processing (Koo

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et al., 2006; Yoshikawa *et al.*, 2006). However, by these means the active ingredient is not preserved due to non-specific cleavage of the glucosidic linkage. An alternative is enzyme degradation, which selectively removes the glucose moiety and is therefore an effective method to preserve the active components.

β-Glucosidases (β-D-glucoside glucohydrolase, EC 3.2.1.21) are members of glycosyl hydrolase families 1 and 3. They mainly catalyze the hydrolysis of the β-glycosidic linkage in various disaccharides, oligosaccharides, alkyl- and aryl-β-D-glucosides (Bhatia *et al.*, 2002; Yang *et al.*, 2009a). In recent years, these enzymes have been widely used in various biotechnological processes. In addition to being utilized for cellulose degradation (Bisaria and Mishra, 1989), β-glucosidases can be used to hydrolyze glycosides to improve their biological activity, including the production of the more active components from isoflavone glycosides or ginsenosides (Hu *et al.*, 2007; Yang *et al.*, 2009a).

For the present study, we assessed *Cistanche tubulosa* for echinacoside and acteoside content, after following the lengthy traditional drying process for stems to be used in preparation of *Cistanche tubulosa* products. We also processed fresh stems with a microwave moisture technique to evaluate this method for preserving echinacoside and acteoside. Finally, echinacoside isolated from Herba Cistanches extract was converted to acteoside by cleaving the β-(1→6)-glucosidic linkage to liberate one molecule of glucose, using specific β-glucosidases (fig. 1). Thus, the present study was performed to improve the harvest of acteoside from the plant *Cistanche tubulosa* via facilitated β-glucosidation.

MATERIALS AND METHODS

Chemicals and enzymes

High performance liquid chromatography (HPLC)-grade methanol was obtained from Sigma-Aldrich (St. Louis, MO, USA). Acteoside and echinacoside were purchased from the National Institute for the Control of

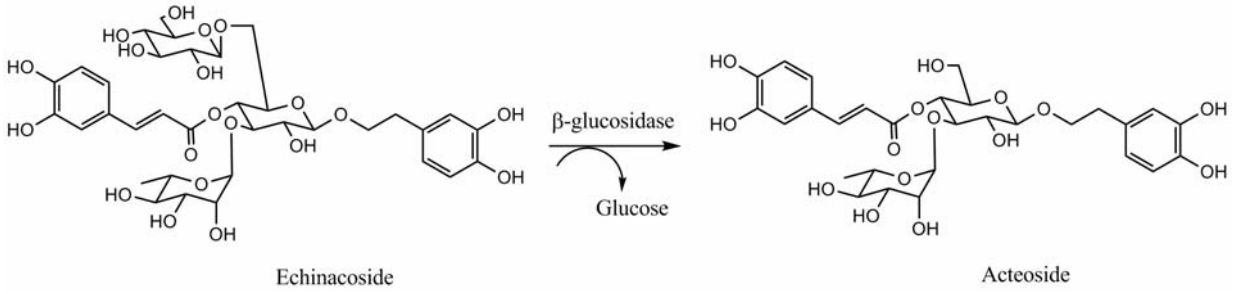

Fig. 1: Transformation pathway of echinacoside to acteoside and their chemical structures.

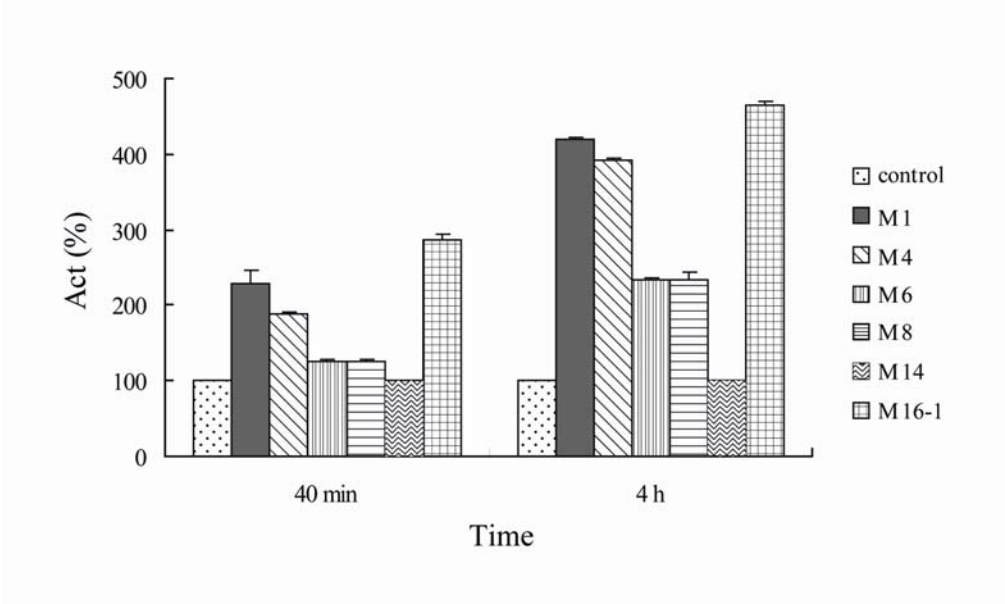

Fig. 2: Production of acteoside via hydrolysis by six crude β-glucosidases. All enzymes except β-glucuronidase (M1) were given at 0.1 U_{pNP}/mL of β-glucosidase activity, and β-glucuronidase was at 0.05 U_{pNP}/mL of β-glucosidase activity. The hydrolyzed substrate was extracted from TL, and incubated at 50°C, pH 5.0 for 40 min and 4 h. Control: TL without hydrolysis by β-glucosidase. Results were expressed as percentages of the beginning value of acteoside, control represented as 100%. Values are reported as mean ± SD for triplicate experiments.

Pharmaceutical and Biological Products (Beijing, China). *p*-Nitrophenyl β-D-glucopyranoside (*p*NPG; catalog no. N7006), and *p*-nitrophenol (*p*NP; catalog no. 241326) were purchased from Sigma-Aldrich. Water was purified using a Millipore Milli-Q Advantage A10 system (Billerica, MA, USA). All other chemicals used were analytical grade reagents unless otherwise stated.

Twelve fresh *C. tubulosa* were collected from different areas of Xinjiang Uygur Autonomous Region, including Moyu, Minfeng, Yutian, and Pishan (table 1). The identities of these plants were confirmed by Prof. Xin Liu who is a botanist. Voucher specimens were deposited at School of Life Sciences, Sun Yat-Sen University. The specimens of *C. tubulosa* were preprocessed in a domestic microwave oven (NN-GT557M, Panasonic Corporation of China, Beijing, China) in a retort pouch at 950 W for 2.5 min per 100 g sample to inactivate enzymes. The samples were then dried at 60 °C and ground into fine powder. A commercially prepared *C. tubulosa* slice, designated TL, was purchased from Sinphar Tian-li (TL) Pharmaceutical (Hangzhou, Zhejiang, China).

The commercial β-glucosidases used in this study are specified below as shown in sample code, product name and source in these order:
M1, β-Glucuronidase, *Helix pomatia*
M14, β-Glucosidase, Almonds
M4, Composite plant hydrolase, *Trichoderma reesei*
M6, Multifect CX GC, *Trichoderma reesei*
M8, Viscozyme L, *Aspergillus aculeatus*
M16-1 and M16-2, Snailase, China white jade snail.

β-Glucuronidase from *Helix pomatia* (M1; Cat. No. G0751) and β-glucosidase from almonds (M14; Cat. No. G0395) were purchased from Sigma-Aldrich. Composite plant hydrolase (M4; containing mainly β-glucosidase, xylanase, and cellulase from *Trichoderma* sp.), was purchased from Hunan Youtell Biochemical (Yueyang, Hunan, China). Multifect CX GC (M6) from *Trichoderma reesei*, a cellulase enzyme complex, was purchased from Danisco US Inc., Genencor Division (Rochester, NY, USA). Viscozyme L (M8), from *Aspergillus aculeatus*, was purchased from Novozymes (Bagsvaerd, Denmark). Snailase (M16-1) was purchased from Fujian Zhangzhou Jin Tian Biotechnology (Zhangzhou, Fujian, China), and snailase (M16-2) was purchased from JingKeHongDa Biotechnology (Beijing, China). All of these commercial enzymes had been produced for food processing.

Preparation and analysis of PhGs from Herba Cistanches

A 0.1 g amount of Herba Cistanches powder was mixed with 10 mL water followed by stirring for 10 min at room temperature. The extraction procedure was repeated three times, and the mixtures were centrifuged at 12,000 ×g for 5 min. The supernatants were filtered through a 0.45 μm filter for the quantification of PhGs (specifically, echinacoside and acteoside) using HPLC. The contents and compositions of echinacoside and acteoside in Herba Cistanches were expressed as g per 100 g of sample.

HPLC was conducted on a Waters liquid chromatograph equipped with a 1525 binary pump and a 2996 photodiode array detector from Waters Corporation (Milford, MA, USA). PhGs were separated and analyzed at 30°C by using a Symmetry-C18 column (4.6 mm × 250 mm, 5 μm) and a Guard column (3.9 mm × 20 mm, 5 μm; Waters). The mobile phase at the flow rate of 1.0 mL/min was made by mixing solvent A (0.5% acetic acid in purified water) and solvent B (methanol) to form a linear gradient of 25-40% B in 0-10 min. The detecting wavelength was set between 210 and 450 nm, and the chromatographic peaks were measured at a wavelength of 330 nm to facilitate the detection of echinacoside and acteoside. Aliquots of 20 μL were directly injected into the HPLC column for the determination. The identifications of echinacoside and acteoside were achieved by comparing their retention time and spectrum with those of known standards. The external standard method was used for the determinations of echinacoside and acteoside. The calibration curve of echinacoside (A = 33874C, regression coefficient (r^2)=0.9999) and acteoside (A = 63469C, r^2 = 0.9988) were established by plotting the peak area (A) against the concentration (C) in the range of 1 - 400 μg/mL.

Determination of the parameters for enzymes

Crude enzyme preparations

β-Glucosidase was extracted from a 0.1 g sample with 5 mL of phosphate-citrate buffer (0.1 M , pH 5.0) for 30 min at room temperature. The enzyme preparation was centrifuged at 12,000×g for 5 min and the supernatant was collected for analysis of enzyme activity.

Enzyme assays

β-Glucosidase activity was measured with *p*NPG as a substrate in pH 5.0, 0.1 M phosphate-citrate buffer (Ribeiro *et al.*, 2007). The reaction mixture, containing 2 mL of 1 mM *p*NPG and 0.5 mL of appropriately diluted enzyme solution, was incubated at 50°C for 20 min. The reaction was then terminated by the addition of 2.5 mL of 0.5 M Na_2CO_3 and the absorption caused by the released *p*NP was measured in a spectrophotometer at 410 nm. A *p*NP (0.02-0.11 μM) calibration curve was previously prepared to determine the enzyme activity. One unit (U_{pNP}) of β-glucosidase activity was defined as the amount of enzyme liberating 1 μmol/min of *p*NP under assay conditions.

Protein concentration determination

Protein was quantified using the protocol of Bradford (Bradford, 1976) using bovine serum albumin as a standard.

Kinetic studies

The activity of commercial β-glucosidase was measured under standard conditions with the concentrations of echinacoside varying from 0.045 to 0.53 mM for M1, M4, M16-1 and M16-2, and varying from 0.42 to 5.0 mM for M6 each in 5 mM ascorbic acid, pH 5.0. The Michaelis-Menten constant (K_m) and maximum reaction velocity (V_{max}) values were calculated by Lineweaver-Burk plot. One unit (U_{Act}) of β-glucosidase activity was defined as the amount of enzyme liberating 1 nmol/min of acteoside at 50 °C, pH 5.0, 30 min.

Conversion of echinacoside to acteoside by commercial β-glucosidases

The Herba Cistanches extract (900 μL, at a final concentration of 0.45 mM echinacoside) was hydrolyzed with 100 μL 1 U_{pNP}/mL of individual β-glucosidase in pH 5.0. The reaction mixture was incubated at 50 °C for 40 min and 4 h, and the reaction was stopped by heating at 75°C for 5 min. The control reaction, consisting of the extraction procedure without enzyme was set up in the same manner. The hydrolyzed sample (20 μL) was directly injected into the HPLC for assessment of the conversion.

STATISTICAL ANALYSIS

Data were expressed as mean ± standard deviation (SD) and analyzed by using Statistical Package for the Social Sciences (SPSS) 16.0 software followed by Student's *t*-test. A *P*-value less than 0.05 was considered statistically significant.

RESULTS

PhGs content of Herba Cistanches

Dried Herba Cistanches powder was extracted with purified water, and the extract was separated and analysed by HPLC. The peaks of echinacoside and acteoside were identified by comparison with commercial standards. Twelve fresh *C. tubulosa* were pretreated by microwave moisture processing; the content of echinacoside ranged from 7.12 to 31.28 g/100 g, and the content of acteoside ranged from 1.71 to 7.48 g/100 g (table 1). The content of echinacoside was 4.16-8.17 times that of acteoside. It has been reported that amount of echinacoside and acteoside varies as the result of differences in habitat, climate, collecting time and soil conditions (Shi *et al.*, 2009).

Kinetic and specificity studies of enzymes

The total and specific activities of each commercial enzyme were measured, as shown in table 2. Data obtained using *p*NPG and echinacoside as active substrates are presented. These results show that the specific activities of M4 were higher than the other commercial enzymes. The specific activity of U_{pNP}/mg protein and U_{Act}/mg protein of M4 were 50.93 and 15.60 fold higher, respectively, than that for M16-1. High β-glucosidase activity (U_{pNP}/g power) was also found in M4, compared with other enzymes.

It must be pointed out that the current kinetic values were derived from a study of crude enzyme preparations extracted from commercial β-glucosidases, and thus the properties shown here may include a combination of isoenzymes and interactions with non-enzymatic proteins. The properties as described below reveal therefore "apparent" quantities (Duangmal and Owusu Apenten, 1999). Furthermore, echinacoside and acteoside as polyphenol antioxidants are very unstable and easily oxidized (Yang *et al.*, 2009b). Therefore we added the typical antioxidant (ascorbic acid) to the extract to protect the echinacoside and acteoside against non-specific oxidative degradation throughout the deglycosylation procedures.

The K_m and V_{max} values of five crude β-glucosidases were determined, using echinacoside as the substrate for five different concentrations. The value for K_m is normally constant for each particular substrate and enzyme, while V_{max} varies according to the substrate concentration. The best enzyme for a particular substrate depends on two factors: strong substrate binding with a low K_m, and high catalytic efficiency with a high V_{max} value (for a fixed enzyme concentration). From the apparent K_m (Table 3), the catalytic efficiency and substrate affinity were the highest for M4, followed by M16-2, M16-1, M1 and M6. Compared to M4, the K_m of M16-1, M1 and M6 were nearly 3.18, 3.73 and 13.27 fold higher, respectively. This may be indicative of their differences in affinity with echinacoside. Therefore, it can be summarized that the β-glucosidase from M4 had substrate-binding sites with high affinity for echinacoside.

Enzyme efficiency for converting echinacoside to acteoside

Six β-glucosidases were compared for hydrolysis of echinacoside in the Herba Cistanches extract. The amount and hydrolysis conditions for each enzyme were based on enzyme specifications from the manufacturers or previous optimization with test products (data not shown). As shown in fig. 2, the extent of conversion of echinacoside to acteoside by M1, M4 or M16-1 β-glucosidases, were found to be more than 95% in 4 h, and the peak for echinacoside was hardly visible. The concentrations of acteoside after the treatment of M1, M4 and M16-1 β-glucosidases were 4.20, 3.91 and 4.65 times higher than before, respectively. On the other hand, only 36.20% and 31.74% of echinacoside was converted into acteoside by M6 and M8, respectively, in the same time. The concentrations of acteoside only increased by 2.34 and 2.33 fold in M6 and M8, respectively (fig. 2). However, the contents of echinacoside and acteoside remained constant in 0.1 U_{pNP}/mL almond β-glucosidase activity at

Table 1: Contents of echinacoside and acteoside from dried *C. tubulosa* samples

No.	Sample code	Sources	Harvesting time[$]	Echinacoside (g/100 g)	Acteoside (g/100 g)
1	B1-1	Moyu	2008.9	18.62 ± 1.41	2.28 ± 0.14
2	B1-2	Moyu	2008.9	25.36 ± 0.58	3.28 ± 0.18
3	B3-1	Moyu	2008.9	31.28 ± 0.86	7.48 ± 0.38
4	MY1-2	Minfeng	2008.9	20.10 ±0.50	4.08 ± 0.14
5	MY2-1	Minfeng	2008.9	19.43 ± 0.20	3.61 ± 0.08
6	MY2-2	Minfeng	2008.9	21.33 ± 0.24	3.36 ± 0.07
7	TL1-1	Yutian	2008.9	25.22 ± 0.13	4.50 ± 0.12
8	TL1-2	Yutian	2008.9	28.90 ± 0.89	5.69 ± 0.35
9	TL2-1	Yutian	2008.9	22.30 ± 0.20	4.73 ± 0.06
10	PT1-2	Pishan	2008.11	11.21 ± 0.06	2.37 ± 0.03
11	PT2-1	Pishan	2008.11	7.12 ± 0.06	1.71 ± 0.04
12	PT2-2	Pishan	2008.11	18.42 ± 0.41	3.36 ± 0.16
13	TL	*C. tubulosa* slice	2008	14.41 ± 0.16	3.28 ± 0.03

Twelve fresh *C. tubulosa* (No. 1-12) were pretreated by microwave moisture processing.
Figures of echinacoside and acteoside are reported as mean ± SD for triplicate experiments.
[$] expressed as year month.

Table 2: Specific activities of commercial β-glucosidases

Enzyme code	U_{pNP}/g powder	U_{pNP}/mg protein	U_{Act}/g powder	U_{Act}/mg protein
M1	166.95 ± 2.82	0.46 ± 0.01	7121.67 ± 167.25	19.63 ± 0.46
M4	552.10 ± 3.62	14.77 ± 0.10	5015.09 ± 104.57	134.16 ± 2.80
M6*	95.36 ± 4.40	2.59 ± 0.12	1293.87 ± 74.94	35.13 ± 2.03
M16-1	117.86 ± 5.54	0.29 ± 0.01	3496.80 ± 141.01	8.60 ± 0.35
M16-2	134.33 ± 0.56	0.32 ± 0.00	3421.39 ± 99.21	8.09 ± 0.23

One unit (U_{pNP}) of β-glucosidase activity was defined as the amount of enzyme liberating 1 μmol/min of *p*NP at 50°C, pH 5.0, 20 min.
One unit (U_{Act}) of β-glucosidase activity was defined as the amount of enzyme liberating 1 nmol/min of acteoside at 50 °C, pH 5.0, 30 min.
* M6 is a liquid crude enzyme, so the unit presents for U/mL sample.

4 h, suggesting that purified almond β-glucosidase (M14) did not have any significant specific deglycosylation activity toward echinacoside. Essentially no hydrolysis was observed in control reactions without enzymes, demonstrating that no active endogenous β-glucosidase was present in the extract of Herba Cistanches. Small amounts of PhGs hydrolyzed in the control extract were possibly due to non-enzyme hydrolysis after 4 h of incubation.

Table 3: Apparent K_m and V_{max} values of commercial β-glucosidases

Enzyme code	K_m (mM)	V_{max} (nmol/min)
M1	0.41 ± 0.01	1.42 ± 0.03
M4	0.11 ± 0.01	1.07 ± 0.02
M6	1.46 ± 0.16	2.59 ± 0.15
M16-1	0.35 ± 0.04	0.71 ± 0.03
M16-2	0.26 ± 0.03	0.36 ± 0.01

The concentrations of echinacoside varying from 0.045 to 0.53 mM for M1, M4, M16-1 and M16-2, and varying from 0.42 to 5.0 mM for M6 each in 5 mM ascorbic acid, pH 5.0, under analysis conditions of 50°C, 30 min. Values are reported as mean ± SD for triplicate experiments.

The present results demonstrated that β-glucosidases from *Trichoderma* and snail were able to convert echinacoside efficiently. Considering the kinetic and specificity studies of the enzymes, we chose M4 as the most appropriate β-glucosidase for converting echinacoside and accumulating a large amount of acteoside. The optimum catalytic conditions of M4 were re-optimized, at 60°C, pH 4.4, 10 mM ascorbic acid. After 2 h of incubation, there was a 4.83 fold increase in the concentration of acteoside, and 97.65% of the echinacoside was hydrolyzed. This was the highest yield in the experiment.

DISCUSSION

Acteoside and echinacoside are polyphenols and are highly susceptible to enzymatic degradation (Nusslein *et al.*, 2000; Wolkart *et al.*, 2004). Examples include the oxidation of polyphenol oxidase and peroxidase (Matsui *et al.*, 2007; Wolkart *et al.*, 2004), and the hydrolysis of esterase (Nusslein *et al.*, 2000). The echinacoside and acteoside content of fresh *Cistanche tubulosa* was reduced during the lengthy traditional drying process. Different processing techniques have been used to

inactivate enzymes of fresh *Cistanche tubulosa* to protect active components. Cai *et al.* established a blanching processing method for *C. tubulosa* pieces: fresh *C. tubulosa* was cut into 4 mm thick slices and put into a 70°C water bath for 6 min (Cai *et al.*, 2007). The echinacoside content of dried *C. tubulosa* pretreated by the blanching processing ranged from 3.18 to 11.99 g/100 g, and that of acteoside from 0.73 to 2.54 g/100 g (Shi *et al.*, 2009). However, when we used microwave moisture processing to inactivate enzymes of fresh *Cistanche tubulosa*, the echinacoside content ranged from 7.12 to 31.28 g/100g, and acteoside was 1.71 to 7.48 g/100g. Thus, the contents of echinacoside and acteoside by microwave moisture processing were higher than that with the blanching process.

Microwaves are able to heat products internally, have greater penetration depth, uniform heating and faster heating rates that potentially improve retention of thermolabile constituents in food (Matsui *et al.*, 2007). When fresh *C. tubulosa* is cut into pieces and comes in contact with oxygen before exposure to inactivating enzymes, the samples turned brown and the constituents are severely degraded by enzymes. Therefore, microwave processing could be more effective for inactivating the enzymes than thermal treatment, and additionally the pretreatment processing established in this study would preserve the active components more effectively than processing by blanching.

Although many β-glucosidases with broad regiospecific bond cleavage activity (1→2, 1→4, 1→6) have been purified from many organisms (Hu *et al.*, 2007), not all of them exhibit hydrolytic activity toward echinacoside. Enzymes preparations with high β-glucosidase activity measured by *p*NPG did not necessarily show echinacoside-degrading activity. Echinacoside is a trisaccharide glycoside which consists of a glucose with a β-(1→6)-glucosidic bond at C6 (Jiang and Tu, 2009). The structure of acteoside is the same as echinacoside but lacking the C6 glucose (fig. 1).

In the present study, purified almond β-glucosidase did not have any significant specific glycosidase activity toward echinacoside. Some β-glucosidases from snail and microorganisms showed highly specific activity towards echinacoside. β-Glucuronidase from *Helix pomatia* was not recognized as a β-glucosidase, but it has been used to liberate lignans (Milder *et al.*, 2004) and isoflavone aglycones (Setchell *et al.*, 2002), indicating that it also contains significant β-glucosidase activity, and therefore echinacoside would be hydrolyzed efficiently. β-Glucosidases from China white jade snail had a wide specificity for various types of β-glucosidic linkage in glucobioses, with the order of hydrolysis rates being gentiobiose (1→6) > cellobioside (1→4) > sophorose (1→2) (Hu *et al.*, 2007). Thus M16-1 and M16-2 could convert echinacoside efficiently. Similarly, when echinacoside was used as the substrate, snailase M16-1 and M16-2 demonstrated different hydrolysis activity.

The cellulase from fungi is a multi-component enzyme complex containing three basic types of enzyme (Bisaria and Mishra, 1989). Industrial hosts for producing cellulases include the saprophytic fungi *Trichoderma reesei*, *Penicillium funiculosum*, *Humicola grisea*, and others (Selig *et al.*, 2008). *Trichoderma* had been proved to be the most promising extracellular cellulase producer. The β-glucosidases of *T. reesei* have been shown to have a broad specificity for many β-glucosides such as gentiobiose, salici, methyl-β-glucoside, sophorose and cellobiose (Jackson and Talburt, 1988). So β-glucosidases from *Trichoderma* had high hydrolyzing efficiency toward echinacoside. However, the multiplicity of β-glucosidases in *T. reesei* and the relationship among these have not been studied at the molecular level (Bisaria and Mishra, 1989). A better understanding of the activity of the *T. reesei* β-glucosidases towards echinacoside will require the purification of the enzyme and kinetic characterization.

Thus, potential application of β-glucosidases for the facilitated conversion of echinacoside to acteoside is suggested for extracts of *Herba cistanches.*

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