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Purification and characterization of baicalin-β-D-glucuronidase hydrolyzing baicalin to baicalein from fresh roots of *Scutellaria viscidula* Bge

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Abstract

The baicalin-β-D-glucuronidase that hydrolyzes the β-D-glucuronide bond of baicalin to form baicalein was isolated from fresh roots of *Scutellaria viscidula* Bge and the enzyme purified and characterized. The enzyme was purified to one band on SDS–polyacrylamide gel electrophoresis and its molecular weight was about 58.4 kDa. The optimum temperature of the baicalin-β-D-glucuronidase was 50 °C, and the optimum pH was 5.0. The baicalin-β-D-glucuronidase was stable at less than 70 °C and pH 4.0–7.0. Ca^{2+} and Zn^{2+} ions have no significant effect on enzyme activity, Mg^{2+} ion has a weakly positive effect and Cu^{2+} ion has a weakly negative effect on enzyme activity, while Fe^{3+} ion inhibits enzyme activity strongly.

Keywords: Purification; Characterization; Baicalin-β-D-glucuronidase; *Scutellaria viscidula* Bge

1. Introduction

Skullcaps are perennial herbs of *Scutellaria* (Labiatae) and their roots have been used as herbal medicines to treat inflammation and infection for several thousand years in China. *Scutellaria baicalensis* Georgi, *Scutellaria viscidula* Bge, *Scutellaria likiangensis* Diels, *Scutellaria amoenac* H. Wright, *Scutellaria rehderiana* Diels and *Scutellaria hypericifolialevl* can be used in traditional Chinese medicine and their main active ingredients are flavonoids including baicalin (baicalein 7-*O*-β-D-glucuronide), baicalein, wogonoside (wogonin 7-*O*-β-D-glucuronide), wogonin, etc. (Fig. 1). Among them, baicalin and baicalein have attracted considerable attention, as they have a variety of interesting activities such as anti-bacterial [1], anti-viral [2], anti-inflammatory [3], anti-cancer [4,5], anti-HIV [6], anti-oxidant and free-radical scavenging [7]. Baicalein is more effective than baicalin in that it is absorbed more slowly and to a lesser extent than baicalein [8,9]. However, it is very difficult to obtain baicalein directly from skullcaps because of its low content (about 0.2–0.5%). A better method of obtaining baicalein is by enzyme hydrolysis from baicalin (Fig. 2), which is present in higher content in skullcaps (about 6–10%).

S. viscidula Bge is widely distributed in mountains of Neimeng, Shanxi and other provinces in China, of which the main active ingredients are similar to that of *S. baicalensis*, and usually used as a substitute for *S. baicalensis* in traditional Chinese medicine [10]. In this paper, baicalin-β-D-glucuronidase was purified and characterized from the fresh roots of *S. viscidula* Bge.

2. Materials and methods

2.1. Materials

The fresh roots of wild *S. viscidula* Bge were collected from Guyang, Neimeng Province, China. The standard

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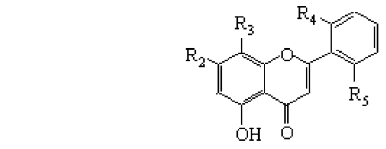
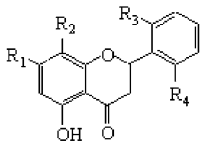
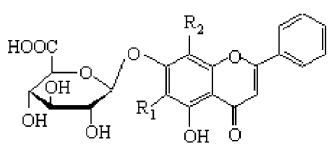


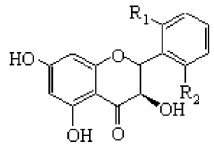
Name	R_1	R_2	R_3	R_4	R_5
baicalein	OH	OH	H	H	H
wogonin	H	OH	OCH_3	H	H
oroxylin-A	OCH_3	OH	H	H	H
chrysin	H	OH	H	H	H
skullcaflavone II	OCH_3	OCH_3	OCH_3	H	H
norwogonin	H	OH	OH	H	H
2',5,7-trihydroxy-6-methoxyflavone	OCH_3	OH	H	OH	H
2',5,6',7-tetrahydroxyflavone	H	OH	H	OH	OH



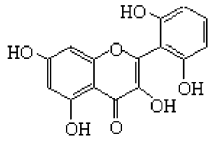
Name	R_1	R_2	R_3	R_4
(2S)-5,7,8-trihydroxy-6-methoxyflavanone	OH	OH	H	H
(2S)- 2',5,6',7-tetrahydroxyflavanone	OH	H	OH	OH
(2S)-2',5.6'-trihydroxy-7-methoxyflavanone-2'-O-β-D-glucopyranoside	OCH_3	H	OH	O-Glc
(2S)-2',5.6'-trihydroxy-7-methoxyflavanone	OCH_3	H	OH	OH



Name	R_1	R_2
baicalin	OH	H
wogonoside	H	OCH_3



Name	R_1	R_2
(2R, 3R)-2',3,5,6',7-pentahydroxyflavanone	OH	OH
(2R, 3R)-3,5,7- trihydroxyflavanone	H	H
(2R, 3R)-2',3,5,7-tetrahydroxyflavanone	OH	H



2',3,5,6',7-pentahydroxyflavone

Fig. 1. Structures of flavonoids in *Scutellaria*.

baicalin (baicalein 7-*O*-β-D-glucuronide), baicalein and wogonin were purchased from Wako Pure Chemical Industries (Osaka, Japan). *p*-Nitrophenyl-β-D-glucuronide (*p*NPGA) and *p*-phenolphthalein-β-D-glucuronide (*p*PGA) were purchased from Sigma. Thin-layer chromatography (TLC) plate was the silica-gel plate (Kieselgel 60 F-254, Merck). All other reagents used in the study were of analytical grade.

2.2. Crude enzyme extraction from roots of S. viscidula

Fresh roots of *S. viscidula* were broken into pieces, washed with acetone to remove baicalin and added to a three-fold volume of 20 mM acetate buffer, pH 5.0. It was extracted at 40 °C for 2 h and the supernatant was obtained by centrifugation for use in enzyme purification.

2.3. Enzyme analysis

Baicalin-β-D-glucuronidase activity was measured using baicalin 1.5 mg/ml in 20 mM acetate buffer, pH 5.0 as the substrate. Enzyme solution 0.1 ml was added to the same volume of baicalin solution and allowed to react at 40 °C for 30 min. The reaction mixture was then heated up to 100 °C for 10 min to stop the enzyme reaction. The baicalein produced was detected by TLC: developing solvent, acetic ether–butanone–formic acid–H_2O (10:7:1:1 v/v), and the baicalein on the silica plate was determined by scanning the TLC spots using a Shimadzu CS-930 [11]. One unit of enzyme activity was defined as the amount of enzyme producing 1 nmol of baicalein per minute.

β-Glucuronidase activity was determined by a colorimetric method using *p*-nitrophenyl-β-D-glucuronide or *p*-phenolphthalein-β-D-glucuronide as substrates [12]. One unit of enzyme activity was defined as the amount of enzyme liberating 1 nmol of *p*-nitrophenol or *p*-phenolphthalein per minute.

2.4. Protein concentration

The concentration of protein was measured according to Bradford [13] using bovine serum albumin as the standard.

2.5. Purification of baicalin-β-D-glucuronidase and estimation of molecular weight

Pellets of $(NH_4)_2SO_4$ were slowly added to the supernatant extracted from fresh roots of *S. viscidula* with shaking

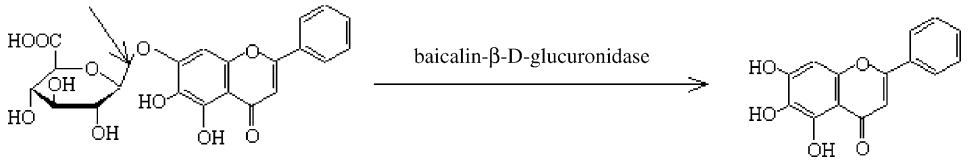


Fig. 2. Producing baicalein from baicalin with baicalin-β-D-glucuronidase.

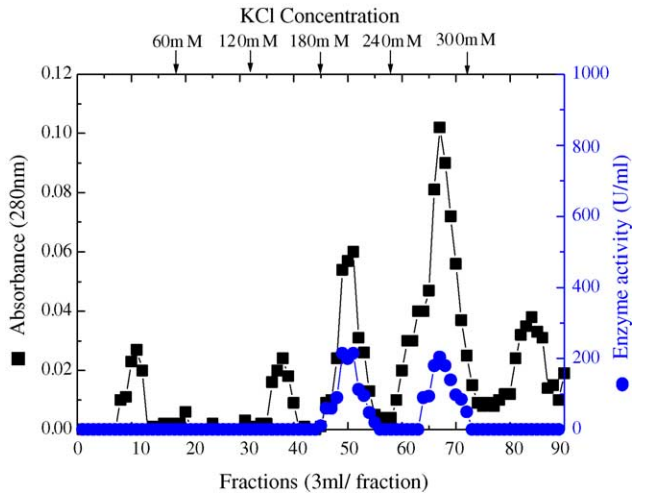


Fig. 3. Purification of baicalin-β-D-glucuronidase on DEAE-cellulose DE-52 column, Ø 1.5 cm × 11 cm; fraction, 3 ml/tube; elution buffer, 60, 120, 180, 240 and 300 mM KCl in 20 mM Tris–HCl buffer (pH 7.4).

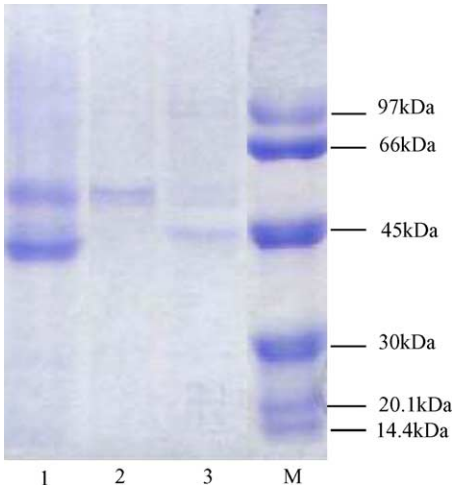


Fig. 4. SDS–polyacrylamide gel electrophoresis of baicalin-β-D-glucuronidase. 1: Crude enzyme deposited by $(NH_4)_2SO_4$; 2: fraction eluted by 180 mM KCl; 3: fraction eluted by 240 mM KCl; M: protein standards α-lactalbumin (14.4 kDa); trypsin inhibitor (20.1 kDa); carbonic anhydrase (30 kDa); ovalbumin (45 kDa); bovine serum albumin (66 kDa); phophorylase b (97 kDa).

to 80% saturation and stored at 4 °C overnight. The mixture was centrifuged to collect the protein precipitate. This crude protein was dissolved in distilled water and dialyzed against 20 mM acetate buffer, pH 5.0. After removing the nondissolved fraction by centrifugation, the enzyme solution was fractionated on a column (Ø 1.5 cm × 11 cm) cm) of DEAE-cellulose DE-52 (Whatman). The column was eluted stepwise with 60, 120, 180, 240 and 300 mM KCl in 20 mM Tris–HCl buffer, pH 7.4. The purified enzyme was dried by freeze-drying and used for the study of enzyme properties. The enzyme purity and molecular weight were estimated by the SDS–polyacrylamide gel electrophoresis method using α-lactalbumin (14.4 kDa), trypsin inhibitor (20.1 kDa), carbonic anhydrase (30 kDa), ovalbumin (45 kDa), bovine serum albumin (66 kDa) and phophorylase b (97 kDa) as standard proteins [14].

3. Results

3.1. Enzyme purification

When the concentration of $(NH_4)_2SO_4$ reached 80% saturation, most of the enzyme was precipitated. The precipitates were centrifuged to collect the protein, and the crude protein was dissolved, dialyzed and fractionated on a DEAE-cellulose column. The result is shown in Fig. 3. The enzyme peak eluted by the step of 180 mM KCl solution had the enzyme activity of hydrolyzing baicalin to baicalein and formed one spot in polyacrylamide gel electrophoresis, confirming that this enzyme was a pure protein. The pure enzyme activity (yield) was 6.2% of total enzyme used in the purification, and the specific activity of pure baicalin-β-D-glucuronidase was 9366 U/mg, an increase of approximately 7.2-fold (Table 1). The enzyme peaks eluted by the step of 240 mM KCl solution also had baicalin hydrolysis activity, but not a pure enzyme.

3.2. Enzyme molecular weight

The purified baicalin-β-D-glucuronidase eluted by the step of 180 mM KCl solution from the DEAE-cellulose column hydrolyzed baicalin to baicalein, formed one band in SDS–polyacrylamide gel electrophoresis (Fig. 4), and its molecular weight was about 58.4 kDa (Fig. 5).

3.3. Some properties of the baicalin-β-D-glucuronidase

For estimating the optimum temperature, the activity was determined by carrying out standard assays at several temperatures. It shows that the optimum temperature of the pure enzyme is 50 °C. To estimate the temperature stability, the residual activity after the incubation of a liquor of the enzyme for 30 min at different temperatures was measured

Table 1
Purification of baicalin-β-D-glucuronidase from fresh roots of *Scutellaria viscidula* Bge

Step	Volume (ml)	Total activity (U)	Total protein (mg)	Specific activity (U/mg)	Yield (%)	Purification
Extraction of fresh roots of *S. viscidula*	550	71062	54.5	1304	100	1
$(NH_4)_2SO_4$ precipitation	23.5	32984	18.5	1783	46.4	1.4
DEAE-cellulose	3	4402	0.47	9366	6.2	7.2

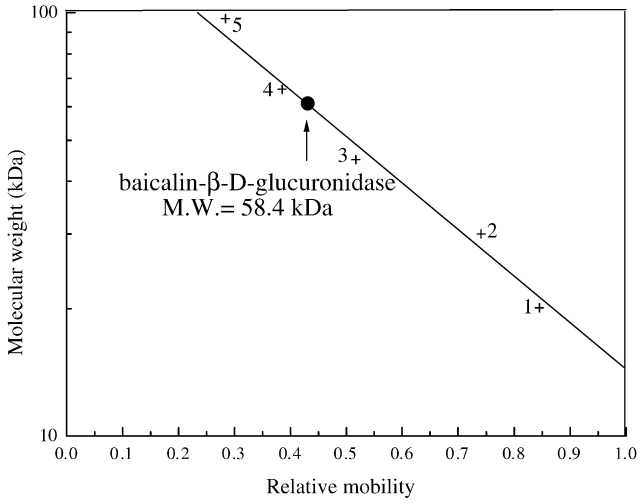


Fig. 5. Molecular weight of baicalin-β-D-glucuronidase in SDS–polyacrylamide gel electrophoresis. 1, Trypsin inhibitor (20.1 kDa); 2, carbonic anhydrase (30 kDa); 3, ovalbumin (45 kDa); 4, bovine serum albumin (66 kDa); 5, phophorylase b (97 kDa).

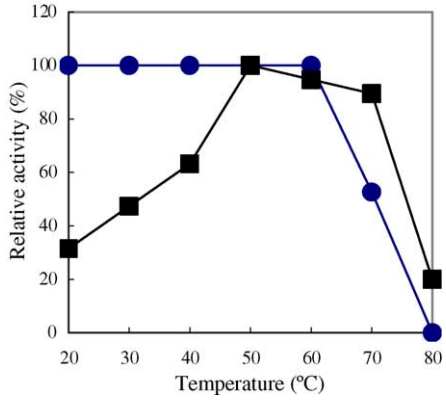


Fig. 6. Optimum temperature and temperature stability of baicalin-β-D-glucuronidase: (■) optimum temperature; (●) temperature stability buffer, 20 mM acetate buffer; pH, 5.0.

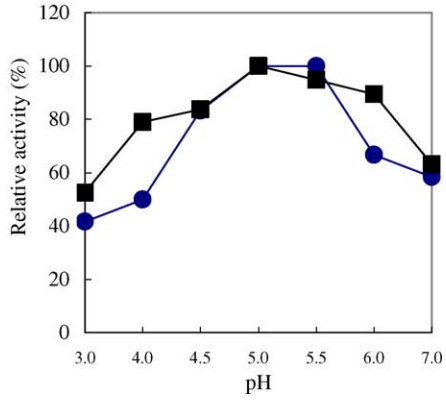


Fig. 7. Optimum pH and pH stability of baicalin-β-D-glucuronidase: (■) optimum pH; (●) pH stability buffer, 20 mM acetate buffer; temperature, 50 °C.

Table 2
Effect of metallic ions on baicalin-β-D-glucuronidase activity

Relative activity (%)					
Concentration (mM)	$CaCl_2$	$MgSO_4$	$FeCl_3$	$ZnSO_4$	$CuSO_4$
0	100	100	100	100	100
1	100	100	71	100	96
5	100	104	57	97	93
10	103	114	50	94	86
50	106	121	40	93	79
100	107	126	29	93	71

under standard conditions. It indicates that the baicalin-β-D-glucuronidase is stable at less than 70 °C (Fig. 6).

For estimating the optimum pH, the activity was determined by carrying out standard assays at several pHs. It shows that the optimum pH of the pure enzyme is 5.0. To estimate the pH stability, the residual activity after the incubation of a liquor of the enzyme for 30 min at different pHs was measured under standard condition. It indicates that the baicalin-β-D-glucuronidase is stable between pH 4.0 and 7.0 (Fig. 7).

In addition, the effect of metallic ions on the baicalin-β-D-glucuronidase was studied. It shows that Ca^{2+} and Zn^{2+} ions have no significant effect on enzyme activity, Mg^{2+} ion has a weakly positive effect and Cu^{2+} ion has a weakly negative effect on enzyme activity, while Fe^{3+} ion inhibits enzyme activity strongly (Table 2).

3.4. Substrate specificity of baicalin-β-D-glucuronidase

The hydrolysis of *p*NPGA and *p*PGA by baicalin-β-D-glucuronidase was carried out in this study. It indicates that the baicalin-β-D-glucuronidase is a flavone-specific β-glucuronidase, which has higher activity of hydrolyzing baicalin (baicalein 7-*O*-β-D-glucuronide) than that of *p*-nitrophenyl-β-D-glucuronide and *p*-phenolphthalein-β-D-glucuronide (Table 3). This result is the same as that of Morimoto [15], and similar to our previous study in ginsenoside-β-glucosidase and glucosidase [16].

4. Discussion

In this study, baicalin-β-D-glucuronidase, i.e., flavone-specific β-glucuronidase was purified and characterized.

Table 3
Hydrolysis of different substrates by baicalin-β-D-glucuronidase substrate concentration, 1 mM; reaction pH, 5.0; reaction temperature, 40 °C; reaction time, 30 min

Substrates	Baicalin-β-D-glucuronidase activity (U/ml)
p-Nitrophenyl-β-D-glucoside	0
p-Nitrophenyl-β-D-glucuronide	55.4
p-Phenolphthalein-β-D-glucuronide	138.6
Baicalein 7-*O*-β-D-glucuronide	1452

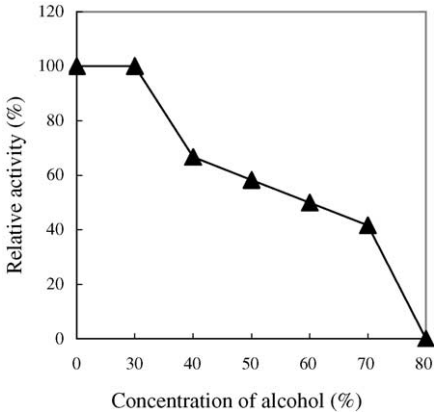


Fig. 8. Effect of alcohol concentration on the activity of baicalin-β-D-glucuronidase.

The baicalin-β-D-glucuronidase hydrolyzed the β-D-glucopyranuronide bond of baicalin to form baicalein and also hydrolyzed wogonoside (wogonin 7-*O*-β-D-glucuronide) to wogonin (data not shown). The molecular weight was about 58.4 kDa. Nearly 100% baicalin was conversed into baicalein by the baicalin-β-D-glucuronidase under the condition of substrate concentration, 1.5 mg/ml; temperature, 50 °C; pH, 5.0; reaction time, 30 min. Because the baicalin-β-D-glucuronidase is very stable, all the purification experiments are carried out at room temperature. It is found that the baicalin-β-D-glucuronidase shows a higher level of activity toward baicalin than towards baicalein and is very stable even in alcohol solution (Fig. 8). Similar researches were carried out with *S. baicalensis* Georgi [17–19]. Levvy's result indicated that baicalinase is specific for β-D-glucopyranuronides and Morimoto et al. purified the β-D-glucuronidase from callus cultures of *S. baicalensis* Georgi and characterized it, which shows a high level of activity towards baicalin than towards baicalein. All this proves that it is easy to transform baicalin into baicalein. It is noticed that there is cell wall peroxidases in skullcaps, which results in baicalein oxidation to 6,7-dehydrobaicalein [20] and accordingly weaken the pharmacological effect. Therefore, it is necessary to insure peroxidases denaturation in transforming baicalin into baicalein. To our knowledge, this is the first report of baicalin-β-D-glucuronidase from *S. viscidula*, which is helpful to the systemic researches of skullcaps and preparation of baicalein. More properties of the baicalin-β-D-glucuronidase and its molecular structure will be further studied in the future.

Acknowledgement

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