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Isolation and characterization of dioscin-α-L-rhamnosidase from bovine liver

Siriguleng Qian*, Hongying Wang, Chunzhi Zhang, Hongshan Yu

School of Biological Engineering, Dalian Polytechnic University, #1 Qinggongyuan, Ganjiangzi District, Dalian 116034, PR China

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ABSTRACT

A novel dioscin-α-L-rhamnosidase was isolated and purified from fresh bovine liver. The activity of the enzyme was tested using diosgenyl-2,4-di-*O*-α-L-rhamnopyranosyl-β-D-glucopyranoside as a substrate. It was cleaved by the enzyme to two compounds, rhamnoses and diosgenyl-*O*-β-D-glucopyranoside. The optimal conditions for enzyme activity were that temperature was at 42 °C, pH was at 7, reaction time was at 4 h, and the substrate concentration was at 2%. Furthermore, metal ions such as Fe^{3+}, Cu^{2+}, Zn^{2+}, Ca^{2+} and Mg^{2+} showed different effects on the enzyme activity. Mg^{2+} acted as an activator whereas Cu^{2+}, Fe^{3+}, and Zn^{2+} acted as strong inhibitors in a wide range of concentrations from 0 to 200 mM. It was interesting that Ca^{2+} played a role as an inhibitor when its concentration was at 10 mM and acted as an activator at the other concentrations for the enzyme. Moreover, the molecular weight of enzyme was determined as 75 kDa.

1. Introduction

It is well-known that α-L-rhamnosidase [EC 3.2.1.40] cleaves terminal α-L-rhamnose specifically from a large number of natural products such as naringin, rutin, quercitrin, hesperidin, diosgene, and terpenyl glycosides [1]. The enzyme has widely occurred in plants, yeasts, bacteria. However, this enzyme was described in only two animal species [2,3].

This enzyme has been a biotechnologically important enzyme due to its applications in a variety of industrial processes including debittering of citrus fruit juices, making prunin from naringin, making L-rhamnose by hydrolysis of natural glycosides containing terminal L-rhamnose, enhancing wine aromas by enzymatic hydrolysis of terpenyl glycosides containing L-rhamnose, eliminating hesperidin crystals from orange juices, converting chloropolysporin B to chloropolysporin C, de-rhamnosylating steroids, such as diosgene, desglucoruscin, ginsenosides Rg2 containing L-rhamnose. Several researchers had demonstrated that those derhamnosylated products had clinical importance [4–8].

Dioscoreae nipponica is a medical plant containing dioscin, a steroidal saponin and its rhizome has been used in Chinese traditional medicine for the treatments of infectious diseases such as bronchial and other respiratory infections [9,10]. It also increases the cardiovascular conditions to treat and reduce risk of heart disease through several mechanisms such as reducing the fat levels in the blood, and protect against cancer. Dioscin had been considered as a main active compound of *D. nipponica*'s rhizome and had been applied in numerous clinical treatments [11–14].

Dioscin is composed of two rhamnose residues, one sapogenin and one glucose residues. The saponin can be hydrolyzed by enzymes produced by intestinal bacteria in vivo, giving products with less rhamnose residues and higher biological activities [15–21]. Although more and more researchers had paid attention to the biotransformation of dioscin, their research was mostly focused on screening of the microbes producing dioscin-α-L-rhamnosidase. On the other hand, a few researches on the enzyme were conducted in animal species.

In the paper, dioscin-α-L-rhamnosidase was isolated from bovine liver and the enzymatic properties were characterized.

2. Experimental

2.1. Materials

Bovine liver was obtained from Dalian Chuming Co. (Dalian city, PR China) and used immediately. The standard diosgenyl-2,4-di-*O*-α-L-rhamnopyranosyl-β-D-glucopyranoside and diosgenyl-*O*-β-D-glucopyranoside were obtained from Academy of Military Medical Sciences, Beijing, China. Diethylaminoethyl cellulose (DEAE-cellulose) was obtained from Pharmacia (Uppsala, Sweden).

Thin layer chromatography (TLC) plate (the silica gel G-60 F_{254}) was purchased from Merck (Darmstadt, Germany); standard proteins including trypsinogen (24 kDa), glyceraldehyde-3-phosphate-dehydrogenase (36 kDa), glutamic dehydrogenase

* Corresponding author. Tel.: +86 411 86323646; fax: +86 411 86307737.
E-mail address: srgl2000@163.com (S. Qian).

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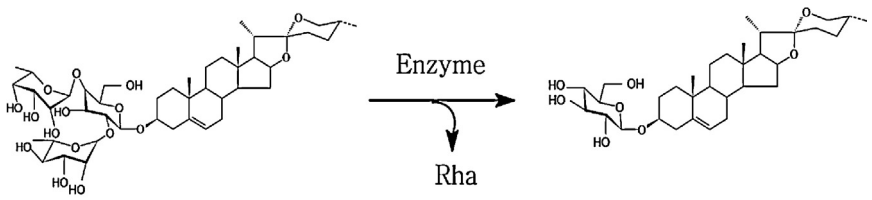

Diosgenyl-2, 4-di-*O*-*α*-L-rhamnopyranosyl-*β*-D-glucopyranoside

Diosgenyl-*O*-*β*-D-glucopyranoside

Fig. 1. Scheme for dioscin hydrolysis by dioscin-*α*-L-rhamnosidase. Rha, rhamnose.

(53 kDa), albumin (66 kDa) and phosphorylase (97 kDa) were obtained from Takara (Takara Biotechnology Co., Ltd. Dalian, China).

2.2. Isolation and purification of dioscin-α-L-rhamnosidase from the bovine liver

The fresh bovine liver was obtained from a slaughterhouse and kept under cold conditions. Five hundreds gram liver was cut into small pieces and homogenized in 1500 mL of 20 mM Tris buffer (pH 7.1). In order to isolate the enzyme-containing fractions, liver homogenate (pH 7.1) was sedimented by centrifugation at 8000 × *g* for 15 min and the pellet was removed. After the enzyme activity had been tested in the cytosol supernatant, it was fractionated by ammonium sulfate. To do this, the cytosol concentration was brought to 35% of the saturation level by adding ammonium sulfate powder, and kept at 4 °C for 12 h. The precipitate was removed by centrifugation as described earlier. Then the concentration of ammonium sulfate in the supernatant was increased to 70% by adding more ammonium sulfate and kept at 4 °C for 12 h for precipitation. The precipitate was dissolved in 50 mL 0.02 M, Tris buffer (pH 6.8). The aliquot of the supernatant were dialyzed for 24 h using 20 mM Tris buffer (pH 7.1) (1: 300) at 4 °C [22].

The dialyzed crude enzyme extract was fractionated by a DEAE-cellulose column (*Φ* 2 cm × 15 cm) at 4 °C. The column was equilibrated with 20 mM Tris–HCl (pH 7.1) at the flow rate of 3 mL/min. The bound proteins were eluted with a linear gradient made of 0–0.6 M KCl. Three milliliter fractions were collected and tested for the enzyme activity [23].

2.3. Enzymatic hydrolysis of dioscin

Same volume of enzyme extract and dioscin solution was incubated at 42 °C for 24 h. 2% of dioscin solution was prepared by dissolving dioscin in 4:1 (v/v) of 20 mM, pH 7.1 Tris–HCl and ethanol. The reaction mixture was stopped and extracted by *n*-butanol saturated water. The butanol layer along with the standard dioscin was applied to TLC plate. It was developed in a TLC tank containing chloroform–methanol–water (70:30:5, v/v/v) mixture and visualized by 10% sulfuric acid [24,25].

2.4. Determination of protein concentration

Protein concentration of the extract was determined followed the method by Lowry et al. using bovine serum albumin as a standard [23].

2.5. Enzyme activity assay

Dioscin-*α*-L-rhamnosidase activity was assayed using the dioscin as a substrate. 0.1 mL substrate (2% dioscin solution) and 0.1 mL enzyme extract were incubated at 42 °C for 4 h, and then 0.2 mLof *n*-butanol saturated water was added into the reaction mixture. Since the hydrolyzed product of dioscin was separated into the butanol layer, the aliquot of the butanol layer was analyzed by TLC As described in Section 2.3. The hydrolyzed dioscin on the TLC plate was determined by scanning the TLC spots using a Shimadzu CS-930. An enzyme unit was defined as 1 mM of the substrate hydrolyzed per hour under specified assay conditions [26] (see Fig. 1).

2.6. Determination of the enzyme molecular weight

The molecular weight of the purified dioscin-*α*-L-rhamnosidase was determined by the sodium dodecylsulfate polyacrylamide gel electrophoresis (SDS-PAGE) method and calibrated by the standard proteins including trypsinogen (24 kDa), glyceraldehyde-3-phosphate-dehydrogenase (36 kDa), glutamic dehydrogenase (53 kDa), albumin (66 kDa) and phosphorylase (97 kDa) [27].

3. Results and discussion

3.1. Isolation and purification of dioscin-α-L-rhamnosidase

Dioscin-*α*-L-rhamnosidase was purified using a DEAE-cellulose column (*Φ* 2 × 15 cm). Among the three peaks that were observed from the fragmentation one showed the enzymatic activity (Fig. 2). This fraction gave a single protein band on SDS-PAGE, confirming the purity of the enzyme (Fig. 3). The specific activity of purified enzyme was 5.9 U/mg, which is 15.7 times higher than crude extract, with purification yield of 4.1% (Table 1).

3.2. Enzymatic hydrolysis of dioscin

Dioscin can be hydrolyzed to give diosgenyl-*O*-*β*-D-glucopyranoside. To confirm that the purified protein is dioscin-*α*-L-rhamnosidase, we performed TLC analysis of dioscin hydrolytes by the purified protein. As shown in Fig. 4, the purified

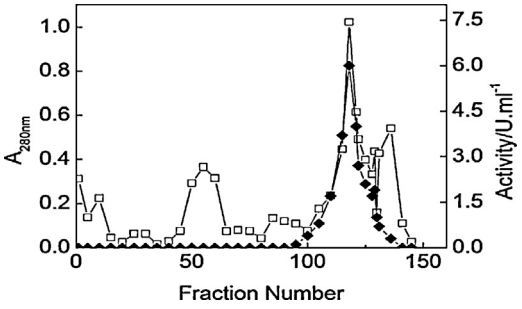

Fig. 2. DEAE-cellulose chromatography of dioscin-*α*-L-rhamnosidase. Column, DEAE-cellulose column (*Φ* 2× 15 cm); solvent, 0–0.6 M KCl in 20 mM pH 7.1 Tris–HCl; fraction, 3 mL; eluting rate, 3 mL/min; -□-, protein; -♦-, enzyme activity.

Table 1
Purification of dioscin-α-L-rhamnosidase from bovine liver.

Purification step	Total protein (mg)	Total activity (U)	Specific activity (U/mg)	Yield (%)	Purification factor (fold)
Crude extract	13,053	4876	0.37	100	1
35–70%ammonium sulfate precipitation	874.6	1651	1.9	33.9	5.1
DEAE-cellulose column chromatography	34.1	199.7	5.9	4.1	15.7

enzyme hydrolyzed dioscin and produced diosgenyl-*O*-*β*-D-glucopyranoside.

3.3. Enzyme properties

Insight into dioscin-α-L-rhamnosidase's substrate specificity is of critical importance for understanding its function in vivo. For this purpose, we examined dioscin, as well as rutin and ginsenoside Re, as possible substrates for dioscin-α-L-rhamnosidase. These compounds contain sugar residues, and linked by glycosidic bond with sapogenin. As shown in Table 2, dioscin-α-L-rhamnosidase from bovine liver showed strong hydrolytic activity on dioscin (42.33 U/mL, +, positive) in vivo, whereas it displays a very low level of activity on rutin (1.51 U/mL, −, negative) and ginsenoside Re (1.42 U/mL, −, negative).

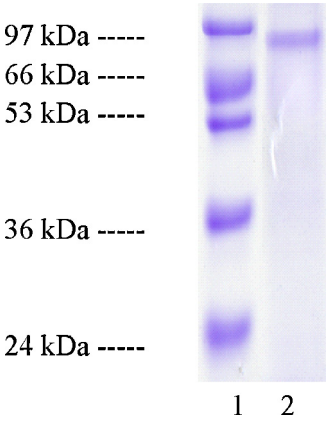

Fig. 3. SDS-polyacrylamide gel electrophoresis of dioscin-α-L-rhamnosidase. Lane 1, standard proteins; Lane 2, dioscin-α-L-rhamnosidase; standard proteins: trypsinogen (24 kDa), glyceraldehyde-3-phosphate-dehydrogenase (36 kDa), glutamic dehydrogenase (53 kDa), albumin (66 kDa), phosphorylase (97 kDa).

Fig. 4. A thin-layer chromatogram of dioscin by the dioscin-α-L-rhamnosidase. The reaction mixture containing 0.1 mL of the substrate and 0.1 mL of the enzyme was incubated for 24 h at 42 °C. Lane 1: standard dioscin; Lane 2: standard diosgenyl-*O*-*β*-D-glucopyranoside; Lanes 3 and 4: two times products from enzymatic transformation.

The effect of pH on the dioscin-α-L-rhamnosidase was studied in pH ranging from 4 to 9. The optimal pH for the hydrolytic reaction was 7 (Fig. 5A). Moreover, thermal activation experiments were carried out by incubating the assay mixture at temperatures ranging from 32 to 57 °C, and the highest activity was exhibited at 42 °C (Fig. 5B). The enzyme activity was examined at a reaction time ranging from 2 to 8 h, and the optimal reaction time was 4 h (Fig. 5C). Various concentrations of dioscin (from 0.1 to 3%) was examined, and 2% substrate resulted in the highest enzyme activity (Fig. 5D).

3.4. Effect of metal ions on dioscin-α-L-rhamnosidase

The influence of different ions on the enzyme activity of dioscin-α-L-rhamnosidase was examined and the results were summarized in Table 3. The presence of Fe^{3+} and Cu^{2+} showed a strong inhibitory effect on the enzyme activity (90% and 95% reduction of activity, respectively). Similar results were obtained using different source α-L-rhamnosidases [28,29]. The addition of Zn^{2+} resulted in almost entirely inhibition of enzyme activity. The α-L-rhamnosidase from *Acrostalagmus luteo albus*, an alkali-tolerant soil fungus [30], and *Pichia angusta* X349, a yeast [28], shown similar results. It was also found that minimal activity was obtained at 10 mM Ca^{2+}, higher salt concentrations were shown a positive effect on the enzyme activity. Similar finding was reported for the α-L-rhamnosidase from *Sphingomonas* sp. R1, a soil bacterium [29], and it was suggested that the enzyme requires Ca^{2+} as a cofactor. The appropriated Mg^{2+} concentration, 10–200 mM, resulted in optimal dioscin-α-L-rhamnosidase activity. However, conversely results were also reported by Rajal et al., they concluded Mg^{2+} has no affected on the α-L-rhamnosidase from *Penicillium ulaiense* [31].

3.5. Molecular weight of dioscin-α-L-rhamnosidase

The molecular weight of the purified dioscin-α-L-rhamnosidase from bovine liver was estimated by comparing its band on SDS-PAGE with standard proteins markers (Figs. 3 and 6), which is calculated to be 75 kDa.

Table 2
α-L-Rhamnosidase hydrolysis substrate.

Enzyme	Dioscin	Rutin	Ginsenoside Re
Dioscin-α-L-rhamnosidase	+	–	–
Rutin-α-L-rhamnosidase	–	+	–
Ginsenoside-α-L-rhamnosidase	–	–	+
α-L-Rhamnosidase (sigma, USA)	–	–	–

+, positive; –, negative.

Table 3
Effect of some metal ions on dioscin-α-L-rhamnosidase activity from bovine liver.

Concentration (mM)	Relative activity (%)				
	0	10	50	100	200
Metal ion					
Fe^{3+}	100	11.8	10.7	10.2	11.4
Ca^{2+}	100	91.6	103.1	109.2	109.1
Mg^{2+}	100	103.0	106.5	114.5	112.2
Zn^{2+}	100	45.8	51.5	74.4	97.0
Cu^{2+}	100	3.2	4.1	5.7	4.3

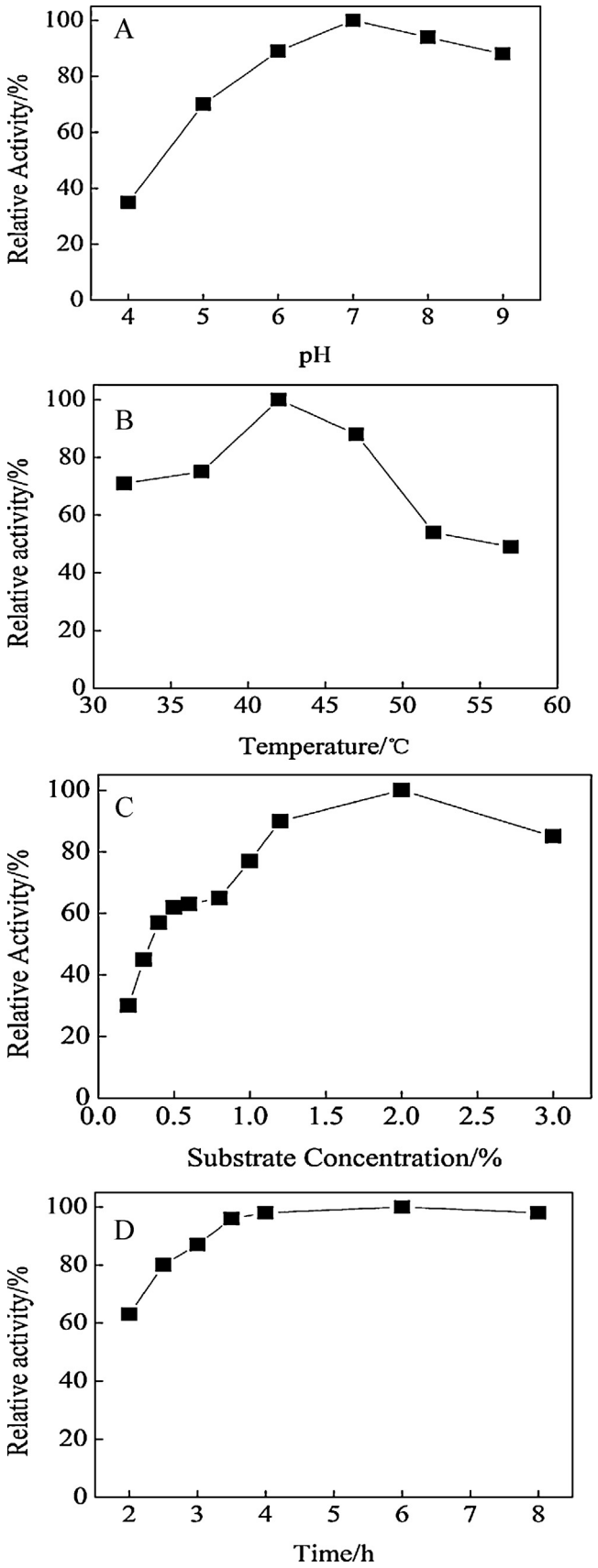

Fig. 5. Optimal reaction conditions for dioscin-α-L-rhamnosidase from Bovine liver. (A) Optimum pH; (B) Optimum temperature; (C) Optimum reaction time; and (D) Optimum substrate concentration.

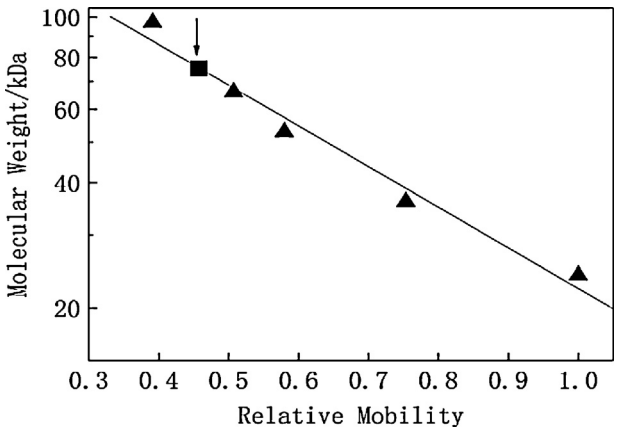

Fig. 6. Molecular weight of dioscin-α-L-rhamnosidase on SDS-polyacrylamide gel electrophoresis. Mobility of dioscin-α-L-rhamnosidase is noted by arrow. The standard proteins used were trypsinogen (24 kDa), glyceraldehyde-3-phosphate-dehydrogenase (36 kDa), glutamic dehydrogenase (53 kDa), albumin (66 kDa) and phosphorylase (97 kDa).

4. Conclusion

The dioscin-α-L-rhamnosidase was purified to electrophoretic homogeneity from bovine liver by ammonium sulfate precipitation, followed by DEAE-cellulose chromatography. In the procedure, from 13,053 mg of total proteins, 34.1 mg of the purified dioscin-α-L-rhamnosidase was obtained with a yield of 4.1% and the purification factor was approximately 15.7-fold (Table 1).

The studies on the influence of metal ions on dioscin-α-L-rhamnosidase activity revealed that Fe^{3+}, Cu^{2+} and Zn^{2+} have inhibitory effect; 10 mM Ca^{2+} negatively affected the enzyme activity, whereas the increased ionic strength showed a positive effect on the enzyme activity; Mg^{2+} significantly promoted the enzyme activity.

The molecular weight obtained for the purified protein corresponds to 75 kDa.

Studies on the effect of temperature on dioscin-α-L-rhamnosidase activity revealed an optimal value of 42 °C (Fig. 5B). The optimal pH value for the hydrolytic reaction was 7 (Fig. 5A), and the optimal reaction time and concentrations of substrate were 4 h and 2% respectively (Fig. 5C and D).

The dioscin-α-L-rhamnosidase displayed high level hydrolytic activity on dioscin, but shown only partial activity on ginsenoside Re and rutin. Ours and other researcher's studies [3,32,33] suggested that the dioscin-α-L-rhamnosidase from bovine liver not only recognize the specific structure of sapogenin but also the type of terminal sugar residue linked to it.

For the first time we report purification of dioscin-α-L-rhamnosidase from bovine liver with the highest yield in a short time. Our study contributes to a better understanding of dioscin-α-L-rhamnosidase and the further usage of the bovine liver as enzyme sources.

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