# Purification and Characterization of Dioscin-α-L-rhamnosidase from Pig Liver

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**Dioscin-α-L-rhamnosidase was isolated, purified and partially characterized from pig liver. The maximum activity was reached at pH 7, 42 °C, 24 h, and 2% of substrate concentration. $Fe^{3+}$ and $Cu^{2+}$ inhibited the enzyme; the ion $Ca^{2+}$ activated it. $Mg^{2+}$ was an inhibitor at 100 mM, but it was an activator at 200 mM. $Zn^{2+}$ could be a weak activator of the enzyme. The molecular weight of dioscin-α-L-rhamnosidase was about 47 kDa as determined by the method of SDS–polyacrylamide gel electrophoresis.**

**Key words** dioscin-α-L-rhamnosidase; enzyme from pig liver; enzyme molecular weight

*Dioscorea nipponica* is a popular herb in China. In Chinese medicine, the rhizome of *Dioscorea nipponica* has been used to prevent bronchial and other respiratory infections as well as viral infections, and to treat rheumatic diseases. The Chinese people also use it to improve cardiovascular conditions to treat and reduce the risk of heart disease through several mechanisms such as reducing the fat levels in the blood, and also to protect against cancer.[1—5] According to the reports,[6] the main active ingredient of the rhizome of *Dioscorea nipponica* is a steroid saponin *i.e.* dioscin. The main dioscin in the rhizome of *Dioscorea nipponica* has three glycosides; the dioscin was hydrolyzed by digestive system enzymes and intestinal bacteria after the administration of medicine.[7,8] Therefore, the transformation of natural products from traditional Chinese medicine to a more active substance is an important study.

Conventional chemical methods such as chemical synthesis, mild acid hydrolysis, or alkaline cleavage for the preparation of minor saponins and saponin metabolites, inevitably produced side reactions, such as epimerization, hydration and hydroxylation,[9—11] but the enzymatic transformation of saponin was mild and direct. Dr. F. X. Jin has successfully isolated an enzyme from some microorganisms to transform the saponin of ginseng,[12] and has industrialized enzymatic transformation production. We found that animal liver such as pig liver contains a high-level concentration of dioscin glycosidase, and its hydrolysis activity is very high. Diosgenyl-2,4-di-*O*-α-L-rhamnopyranosyl-β-D-glucopyranoside (dioscin) was hydrolyzed to diosgenyl-*O*-β-D-Glc by the enzyme from pig liver (Fig. 1). The enzyme, *i.e.* dioscin-α-L-rhamnosidase or dioscin glycosidase, was purified from pig liver and its kinetic characteristics were systemically investigated.

## Experimental

**Materials** Fresh pig liver was obtained from a local abattoir and used immediately. DEAE-Cellulose was obtained from Pharmacia. The standard dioscin and diosgenyl-*O*-β-D-Glc was obtained from Prof. Baiping Ma of the Academy of Military Medical Sciences, Beijing, China. Thin-layer chromatography (TLC) was conducted on silica gel G-60 $F_{254}$ (Merck). Standard proteins such as trypsinogen (24 kDa), glyceraldehyde-3-phosphate-dehydrogenase (36 kDa), glutamic dehydrogenase (53 kDa), albumin (66 kDa) and phosphorylase (97 kDa) were Sigma products (Sigma Chemical Co., St. Louis, MO, U.S.A.).

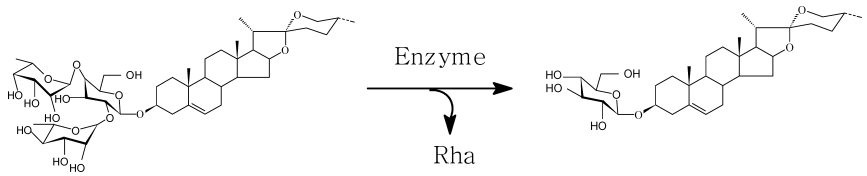
**Enzyme Preparation** Fresh pig livers were homogenized (1 : 3, w/v) in a 20 mM pH 7.2 Tris–HCl buffer (4 °C), and the supernatant was brought to 45% saturation with ammonium sulfate, then kept at 4 °C for 18 h, and centrifuged at 8000 rpm, 4 °C, for 20 min to remove the precipitated protein. Then, the supernatant was brought to 65% saturation with ammonium sulfate, and stored at 4 °C overnight. The precipitated protein was collected by centrifugation, suspended in distilled water and dialyzed against 20 mM pH 7.2 Tris–HCl buffer, and the non-dissolved protein was removed to yield the enzyme solution. The enzyme solution was freeze-dried.

The crude enzyme protein was dissolved in 20 mM pH 7.2 Tris–HCl buffer and subjected to a DEAE-cellulose column (2×15 cm). The bound proteins were eluted with 0—0.6 M KCl in 20 mM pH 7.2 Tris–HCl buffer; fraction, 3 ml; elution rate, 3 ml/min.[13]

**Enzyme Hydrolysis of Dioscin** The dioscin solution in 20 mM pH 7.2 Tris–HCl buffer was added to the enzyme to react at 37 °C for 24 h, followed by the addition of *n*-butanol saturated water. The *n*-butanol layer was concentrated to dryness in vacuum prior to TLC: plate, silica gel G-60 $F_{254}$; solvent, chloroform–methanol–water (70 : 30 : 5, v/v/v); detection, 10% sulfuric acid.[14—16]

**Protein Determination** The concentration of protein was measured by the Folin phenol reagent method using bovine serum albumin as a standard.[17]

**Enzyme Analysis** Dioscin-α-L-rhamnosidase was assayed using dioscin as the substrate. The assay mixture containing 0.1 ml of the substrate (2%




Diosgenyl-2, 4-di-*O*-α-L-Rhal-β-D-Glc Diosgenyl-*O*-β-D-Glc

Fig. 1. Dioscin Hydrolysis

Rha: rhamnopyranosyl; Glc: glucopyranoside.

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dioscin solution) and 0.1 ml of the enzyme was incubated at 37 °C for 24 h. The reaction was stopped by the addition of 0.2 ml of *n*-butanol saturated water. The hydrolyzed product of dioscin was removed to the butanol layer, and an aliquot of the butanol layer was carried out by TLC: solvent, chloroform–methanol–water (70 : 30 : 5, v/v/v). The amount of dioscin produced on the silica gel G-60 $F_{254}$ plate was determined by scanning the TLC spots using a Shimadzu CS-930. One unit of the enzyme activity equals 1 mM of the substrate hydrolyzed per hour.[18,19]

**Enzyme Molecular Weight** Purified dioscin glycosidase was used to determine the molecular weight of the enzyme by SDS–polyacrylamide gel electrophoresis (SDS–PAGE). SDS–PAGE was also carried out for standard proteins under the same conditions. The molecular weight of dioscin glycosidase was determined by plotting the log of the molecular weights of the standard proteins.[20] The standard proteins were trypsinogen (24 kDa), glyceraldehyde-3-phosphate-dehydrogenase (36 kDa), glutamic dehydrogenase (53 kDa), albumin (66 kDa) and phosphorylase (97 kDa).

## Results and Discussion

**Enzyme Purification** Dioscin glycosidase purification was carried out with a typical DEAE-cellulose column. The fractions showed two peaks of protein, but only the 40-fraction showed enzyme activity (Fig. 2). The 40-fraction enzyme was one spot in SDS–PAGE (Fig. 3). In the purification, the specific activity was 5.3 U/mg; the enzyme specific activity was increased 12.3 times and the yield was 3.9% as shown in Table 1.

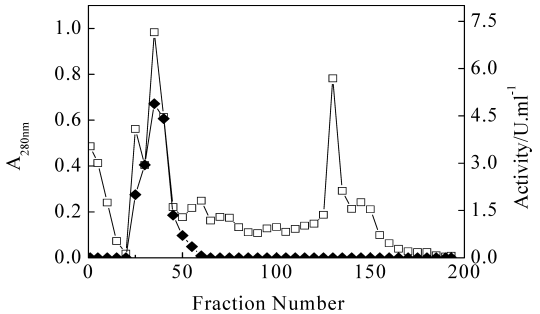


Fig. 2. DEAE-Cellulose Chromatography of Dioscin-$\alpha$-L-rhamnosidase

Column, DEAE-cellulose column (2×15 cm); solvent, 0—0.6 M KCl in 20 mM pH 7.2 Tris–HCl buffer; fraction, 3 ml; eluting rate, 3 ml/min; □, protein; ◆, enzyme activity.

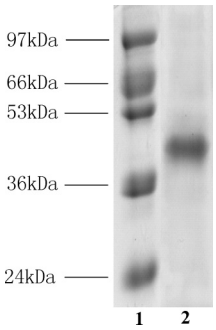


Fig. 3. SDS–Polyacrylamide Gel Electrophoresis of Dioscin-$\alpha$-L-rhamnosidase

1: standard proteins; 2: dioscin-$\alpha$-L-rhamnosidase; standard proteins: trypsinogen (24 kDa), glyceraldehyde-3-phosphate-dehydrogenase (36 kDa), glutamic dehydrogenase (53 kDa), albumin (66 kDa), phosphorylase (97 kDa).

**Enzyme Hydrolysis of Dioscin** The result of dioscin hydrolyzed by enzymes from pig liver is shown in Fig. 4. As seen in the figure, the dioscin was hydrolyzed into a product showing the same *Rf* value as the diosgenyl-*O*-$\beta$-D-Glc on the TLC, and this transformation of dioscin was more than 90%. It showed that pig liver contained dioscin glycosidase and its activity of transforming dioscin was very high. The identification of a spot with a lower *Rf* value on the TLC is the subject of a future study.

**Enzyme Properties** The enzymes hydrolyzing the substrates are shown in Table 2. Purified dioscin-$\alpha$-L-rhamnosidase from pig liver hydrolyzed the $\alpha$-L-rhamnoside of dioscin, but did not hydrolyze that of ginsenoside Re or rutin. These results suggest that the dioscin-$\alpha$-L-rhamnosidase from pig liver is a special dioscin-$\alpha$-L-rhamnosidase and a higher specificity enzyme. The dioscin was not hydrolyzed by the $\alpha$-L-rhamnosidase (Sigma), rutin-$\alpha$-L-rhanmosidase[21] or ginsenoside-$\alpha$-L-rhanmosidase.[22]

The enzyme from pig liver hydrolyzed two rhamnosides of dioscin to diosgenyl-*O*-$\beta$-D-Glc. The effect of pH value on enzyme activity was examined at pH 4, 5, 6, 7, 8 and 9. The highest activity of dioscin glycosidase was reached at pH 7 (Fig. 5). The enzyme activity was stable from 32 to 52 °C, and the highest enzyme activity was at 42 °C, which is thus the optimum temperature (Fig. 6). The effect of the reaction time on the enzyme activity was examined at 6, 12, 18, 24, 30, and 36 h. The reaction lasted for 24 h, at which point it reached the highest activity (Fig. 7). The substrate concentration of 2% produced the highest enzyme activity (Fig. 8).

**Effect of Some Metal Ions on Dioscin-$\alpha$-L-rhamnosidase** The effects of some metal ions on dioscin glycosidase are shown in Table 3. As shown above, $Fe^{3+}$ and $Cu^{2+}$ gave

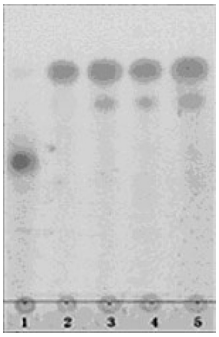


Fig. 4. TLC of Dioscin Hydrolysis by Enzyme

The reaction mixture containing 0.1 ml of the substrate and 0.1 ml of the enzyme was incubated for 24 h at 37 °C. The reaction was stopped by the addition of 0.2 ml of *n*-butanol saturated water. An aliquot of the *n*-butanol layer was applied on TLC: solvent, chloroform–methanol–water (70 : 30 : 5, v/v/v); detection, 10 % sulfuric acid. 1: standard dioscin; 2: standard diosgenyl-*O*-$\beta$-*D*-Glc; 3, 4, and 5, three products from the enzyme reaction.

Table 1. Purification of Dioscin-$\alpha$-L-rhamnosidase from Pig Liver

| Purification step | Total protein (mg) | Total activity (U) | Specific activity (U/mg) | Yield (%) | Purification |
|---|---|---|---|---|---|
| Crude extract | 8598 | 3690 | 0.4 | 100 | 1.0 |
| 45—65% ammonium sulfate precipitation | 678.4 | 782 | 1.2 | 21.2 | 2.7 |
| DEAE-cellulose | 27.5 | 144.8 | 5.3 | 3.9 | 12.3 |

Table 2. $\alpha$-L-Rhamnosidase Hydrolysis Substrate

| Enzyme | Dioscin | Ginsenoside Re | Rutin |
|---|---|---|---|
| Dioscin-$\alpha$-L-rhamnosidase | + | − | − |
| $\alpha$-L-Rhamnosidase (Sigma) | − | − | − |
| Rutin-$\alpha$-L-rhanmosidase[21] | − | − | + |
| Ginsenoside-$\alpha$-L-rhanmosidase[22] | − | + | − |

+, positive; −, negative.

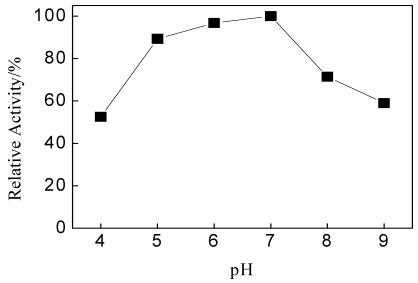


Fig. 5. Optimum pH of Dioscin-$\alpha$-L-rhamnosidase

The substrate dioscin, 20 mg/ml; enzyme, 10 U/ml; reaction temperature, 37 °C.

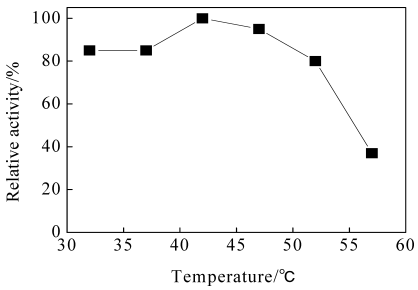


Fig. 6. Optimum Temperature of Dioscin-$\alpha$-L-rhamnosidase

The substrate dioscin, 20 mg/ml; enzyme, 10 U/ml; reaction time, 24 h.

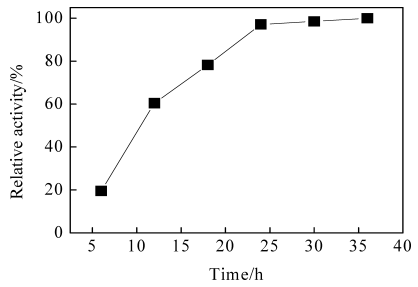


Fig. 7. Optimum Reaction Time of Dioscin-$\alpha$-L-rhamnosidase

The substrate dioscin, 20 mg/ml; enzyme, 10 U/ml; reaction temperature, 37 °C.

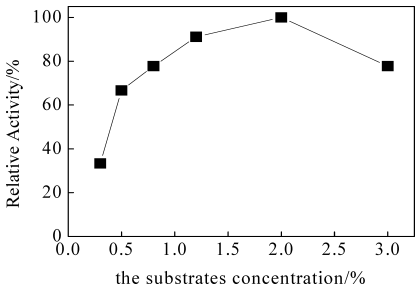


Fig. 8. Optimum Concentration of Substrate of Dioscin-$\alpha$-L-rhamnosidase

Enzyme, 10 U/ml; reaction temperature, 37 °C.

Table 3. Effect of Some Metal Ions on Activity of Dioscin-$\alpha$-L-Rhamnosidase from Pig Liver

| | Relative activity/% | | | | |
|---|---|---|---|---|---|
| Concentration/mM | 0 | 10 | 50 | 100 | 200 |
| Metal ion | | | | | |
| $Fe^{3+}$ | 100 | 11.5 | 14.1 | 15.4 | 15.4 |
| $Ca^{2+}$ | 100 | 113 | 114 | 115 | 118 |
| $Mg^{2+}$ | 100 | 64.1 | 77 | 94.9 | 109 |
| $Zn^{2+}$ | 100 | 108 | 109 | 109 | 110 |
| $Cu^{2+}$ | 100 | 6.4 | 3.9 | 2.6 | 6.4 |

The substrate dioscin, 20 mg/ml; enzyme, 10 U/ml; at 37 °C for 24 h.

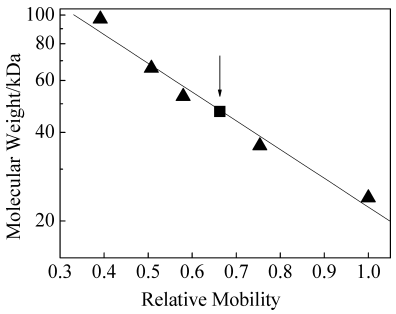


Fig. 9. Molecular Weight of Dioscin-$\alpha$-L-rhamnosidase on SDS–Polyacrylamide Gel Electrophoresis

Mobility of dioscin-$\alpha$-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).

approximately 85% inhibition of the enzyme, and the ion $Ca^{2+}$ activated the enzyme. $Mg^{2+}$ was an inhibitor at 100 mM, but it was an activator at 200 mM. $Zn^{2+}$ could be a weak activator to the enzyme.[23]

**Molecular Weight of Dioscin-$\alpha$-L-rhamnosidase** The molecular weight of dioscin glycosidase was estimated by SDS–PAGE (Figs. 3, 9). The purified enzyme was a spot in the electrophoresis showing that the enzyme was a pure protein, and the molecular weight was about 47 kDa.

The pig liver contained dioscin glycosidase *i.e.*, dioscin-$\alpha$-L-rhamnosidase. The purified dioscin-$\alpha$-L-rhamnosidase from pig liver hydrolyzed the dioscin into the diosgenyl-*O*-$\beta$-D-Glc. However, it did not hydrolyze ginsenoside Re or rutin. Dioscin-$\alpha$-L-rhamnosidase is a higher specificity enzyme.

**Acknowledgements** We thank Dr. Sun Q. Y. for suggestions. This work was financially supported by the National Natural Science Foundation of China (NSFC), Science & Technology Department of Liaoning Province, and the Science & Technology Department of Dalian City, China.

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