A novel dioscin-glycosidase that specifically hydrolyzes multi-glycosides, such as 3-O-α-L-(1→4)-rhamnopyranosyl-3-O-α-L-(1→2)-rhamnopyranosyl-3-O-α-L-(1→4)-arabinoside, and β-D-glucose, on diosgenin was isolated from the Absidia sp.d38 strain, purified, and characterized. The molecular mass of the new dioscin-glycosidase is about 55 kDa based on SDS–PAGE. The dioscin-glycosidase gradually hydrolyzes either 3-O-α-L-(1→4)-Rha or 3-O-α-L-(1→2)-Rha from dioscin into 3-O-α-L-Rha-β-D-glucopyranosyl-diosgenin, further rapidly hydrolyzes the other α-L-Rha from 3-O-α-L-Rha-β-D-glucopyranosyl-diosgenin into the main intermediate products of 3-O-β-D-glucopyranosyl-diosgenin, and subsequently hydrolyzes these intermediate products into aglycone as the final product. The enzyme also gradually hydrolyzes 3-O-α-L-(1→4)-arabinosyl-3-O-α-L-(1→2)-rhamnopyranosyl- and β-D-glucose from [3-O-α-L-(1→4)-Ara, 3-O-α-L-(1→2)-Rha]-β-D-glucopyranosyl-diosgenin into diosgenin as the final product, exhibiting significant differences from previously reported glycosidases. The optimal temperature and pH for the new dioscin-glycosidase is 40°C and 5.0, respectively. Whereas the activity of the new dioscin-glycosidase was not affected by Na+, K+, and Mg2+ ions, it was significantly inhibited by Cu2+ and Hg2+ ions, and slightly affected by Ca2+ ions.

**Keywords:** Dioscin, dioscin-glycosidase, hydrolyzing multi-glycosides

**Dioscorea nipponica** is a popular herb in China and widely used in traditional Chinese medicine, functional foods, and cosmetics. The Chinese agricultural name for *Dioscorea nipponica* is Huangjiang, and the rhizome of *Dioscorea nipponica* is used to prevent rheumatic diseases, viral infection, and bronchitis [17]. One of the major physiologically active ingredients in *Dioscorea nipponica* is dioscin, which belongs to the family of steroidal saponins. Dioscin has been proven to be physiologically active, improving the cardiovascular function and exhibiting antitumor [11, 13], anticancer [3, 10], anti-diabetic [12], and antiviral [6] activities. It is also an important ingredient for the chemical synthesis of steroidal drugs.

The main dioscin in the rhizome of *Dioscorea nipponica* is 3-O-α-L-rhamnopyranosyl-(1→4), [α-L-rhamnopyranosyl-(1→2)]-β-D-glucopyranosyl-diosgenin (i.e., 3-O-α-L-Rhap-(1→4), [α-L-Rhap-(1→2)]-β-D-Glc-p-diosgenin), which contains three glycosides [14, 15]. In general, saponins in their natural form (i.e., dioscin in *Dioscorea nipponica*) cannot be directly absorbed and utilized by the human body. After oral intake of *Dioscorea nipponica*, the glycosides of dioscin are hydrolyzed by digestive enzymes and/or intestinal bacteria into low-sugar-saponin and aglycone, which are absorbed slowly in the gastrointestinal tract to exhibit physiological activity [1, 8]. Therefore, the modification of natural saponins by enzymes in vitro to produce more active second saponins that can be easily absorbed and utilized by the human body would add great value to functional foods and medicines made from herbs such as *Dioscorea nipponica*.

Accordingly, to obtain physiologically active saponins that can be easily absorbed by the human body, the current authors previously reported on saponin-glycosidases, isolated and extracted from microorganisms, plants, and livers, that hydrolyze the saponin-sugar moiety to produce more active low-sugar-moiety saponins [9, 14, 18, 19]. Three types of enzymes hydrolyzing dioscin-glycosides were previously reported: dioscin-α-L-rhamnopyranosidase from pig liver [14] and...
dioscinase from a microorganism [9]. The enzyme from pig liver [14] hydrolyzes 3-O-α-L-(1→2)-rhamnopyranoside and 3-O-α-L-(1→4)-rhamnopyranoside from dioscin to produce 3-O-β-D-glucopyranosyl-diosgenin, whereas the dioscinase from the microorganism (sp.s00c strain) [9] hydrolyzes 3-O-α-L-(1→2)-Rhap and 3-O-α-L-(1→4)-Rhap from dioscin to produce 3-O-β-D-glucopyranosyl-diosgenin; it also hydrolyzes 3-O-α-L-(1→2)-Rhap and 3-O-β-L-(1→3)-GlcP from 3-O-α-L-(1→2)-Rhap, [3-O-β-L-(1→3)-GlcP]-β-GlcP-diosgenin to produce 3-O-β-D-glucopyranosyl-diosgenin. Feng et al. [4, 5] also reported a glucoamylase with steroidal saponin-rhamnosidase activity from a Curvularia lunata strain. This enzyme is specific to the terminal α-1,2-linked rhamnosyl residues of the sugar chain at the 3-O-position of dioscin; that is, the enzyme only hydrolyzes the α-L-(1→2)-rhamnoside from dioscin to produce 3-O-α-L-Rhap-(1→4)-β-D-Glc-diosgenin.

Therefore, this study isolated, purified, and characterized a new dioscin-glycosidase from the Absidia sp.d38 strain that specifically and gradually hydrolyzes either 3-O-α-L-(1→4)-Rha or 3-O-α-L-(1→2)-Rha from dioscin to produce 3-O-α-L-Rha-β-D-Glc-diosgenin, rapidly hydrolyzes the other α-L-Rha from 3-O-α-L-Rha-β-D-Glc-diosgenin into the main intermediate products of 3-O-β-D-Glc-diosgenin, and subsequently hydrolyzes these intermediate products into aglycone as the final product (Fig. 1).

**Materials and Methods**

**Materials**
The Absidia sp.d38 strain isolated from Chinese traditional koji (Daqu in Chinese) [19] was obtained from the Food and Fermentation Culture Collection at Dalian Polytechnic University, Dalian, P. R. China. The herb, Dioscorea nipponica, was purchased from Xianyang, Shaanxi Province, P. R. China, and the dioscin extracted following existing procedures [15]. Standard dioscin, diosgenin, 3-O-[α-L-Rha-(1→2), α-L-Ara-(1→4)]-β-D-Glc-diosgenin, 3-O-[α-L-Rha-β-D-Glc-diosgenin, and 3-O-β-D-Glc-diosgenin were all obtained from Prof. Baojin Ma (Academy of Military Medical Science, Beijing, P. R. China) and Sino-Herb Bio-Tech Co. Ltd, Xian, P. R. China. Silica gel (Kiesel gel 60 F-254, Merck) was used as the thin-layer chromatography (TLC) plate, and the protein molecular markers were from GE Healthcare Life Science Products, LMW (14,000–97,000). All the other reagents in the study were of analytical grade and used without further purification.

**Microorganism Culturing**
The Absidia sp.d38 strain was cultured at 28–30°C with shaking in a medium (200 ml in 1,000 ml conical flask) containing 5% malt extract and 0.75% plant extract from Dioscorea nipponica for 84–96 h. The maltose, cell growth, and enzyme activity in the fermentation were determined following existing procedures [7].

**Crude Enzyme Extraction**
The culture was centrifuged to remove the cells, and (NH₄)₂SO₄ was then slowly added to the cell-free culture with stirring to 40% saturation and the mixture stored at 4°C for 4 h. After removing the protein precipitated by the centrifugation, more (NH₄)₂SO₄ was added to 75% saturation and the mixture stored at 4°C overnight. Thereafter, the mixture was centrifuged to harvest the crude enzyme, which was then dialyzed against a 0.02 M acetic acid buffer (pH 5.0) and subsequently diluted to 1/20 (v/v) with the 0.02 M acetic acid buffer (pH 5.0). The non-dissolved fraction was removed by centrifugation, and the resulting crude enzyme solution subjected to further enzyme purification.

**Purification of New Dioscin-Glycosidase**
Ten ml of the crude enzyme solution was eluted on a DEAE-cellulose DE-52 (Whatman) column (92.0 cm × 11 cm) and the proteins were

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**Fig. 1.** Action patterns of novel dioscin-glycosidase in dioscin hydrolysis.
fractionated stepwise with 0.06, 0.12, 0.18, 0.24, 0.3, 0.4, 0.5, and 0.6 M KCl in a 0.02 M acetate buffer (pH 5.0, 3.0 ml/fraction). The enzymatic activity of each fraction was then evaluated based on the hydrolysis of dioscin, and the fractions that exhibited hydrolyzing activity were respectively dialyzed against a 0.02 M acetate buffer (pH 5.0), freeze-dried, and dissolved in 1/10 (v/v) distilled water. Vertical slab polyacrylamide gel electrophoresis was also used for further purification of the special dioscin-glycosidase. The resulting enzyme band on the vertical slab polyacrylamide gel was cut and mashed in an acetate buffer, and the non-dissolved materials were removed by centrifugation, leaving a pure enzyme solution that was evaluated for its molecular mass and enzymatic properties. The enzyme protein purity was also examined by HPLC using a C8 column.

Assays of Enzymatic Activity
The enzymatic activity of the dioscin-glycosidase was assayed using 2.0 mg/ml dioscin in a 0.2 M acetate buffer (pH 5.0) as the substrate. A dioscin solution (0.1 ml) was added to the same volume of the enzyme solution and allowed to react at 40°C for 30 h. Next, 0.2 ml of n-butanol saturated by water was added to the reaction mixture to stop the reaction. The reaction product in the n-butanol layer was then analyzed by TLC with chloroform:methanol: 

HPLC Method
HPLC (Waters 2695 Separations Module with Waters 2996 Photodiode Array Detector) was used to examine the enzyme protein purity and analyze the enzymatic reaction product.

A C-18 Hypersil 5 μm ODS2 (Φ4.6×250 mm) column was used to analyze the enzymatic reaction product, where the measuring wavelength was 203 nm and the injected volume was 10 μl. The mobile phase was A (acetonitrile) and B (water): 0 to 2 min, A from 0% to 70%; 2–10 min, A from 70% to 80%; 10–20 min, A from 80% to 90%; 20–40 min, A 90%; and 40–60 min; the column was eluted by 100% A. The enzymatic reaction product sample used for the HPLC was obtained through pretreatment of the reaction product with an AB-8 Diaion resin column. One ml of a dioscin enzyme reaction mixture was separated and collect the reaction products. These products were dried and phosphorylase b (97 kDa) as the standard proteins under the same conditions [16]. The enzyme molecular mass was determined from the standard curve constructed using the standard proteins.

Determination of Protein Concentration
The protein concentration was measured using the Folin reagent method of Bradford with bovine serum albumin as the standard [2].

Assays of Effects of pH, Temperature, and Reaction Time on Dioscin-Glycosidase Reaction
To determine the optimal pH, temperature, and reaction time for the new dioscin-glycosidase, various pHs, temperatures, and reaction times were investigated for the enzymatic reaction, and the products analyzed by TLC. The optimal pH for the special dioscin-glycosidase was determined at 40°C with different buffers at 0.2 M. An acetate buffer and phosphate buffer were used for pH 4.0–5.0 and 6.0–8.0, respectively. The optimal temperature was determined between 30°C and 80°C using a standard enzymatic assay at pH 5.0 (0.2 M acetate buffer), and the optimal reaction time was examined between 3 h and 39 h at 40°C and pH 5.0 (0.2 M acetate buffer).

RESULTS

Enzyme Fermentation
To examine the production behavior of dioscin-glycosidase during the fermentation of the Absidia sp.d38 strain, the cell growth, enzyme production, and maltose reduction were all measured, as shown in Fig. 2.

The highest dioscin-glycosidase production by the Absidia sp.d38 strain occurred after fermentation for 84 to 96 h, at which point the enzyme production reached about 13 U/ml. Even after 96 h, the enzyme production was still maintained on a high level. Meanwhile, the maltose rapidly reduced from 60 to 84 h, and became stable after 96 h, whereas the cell concentration rapidly increased from 60 to

![Fig. 2. Enzyme fermentation behavior.](image)
84 h, and became stable after 84 h, indicating that the maltose was quickly utilized in the cell growth to produce the enzyme during the fermentation process. Thus, since the enzyme production was found to be closely related to the cell growth, the enzyme fermentation time was defined as 84 to 96 h in the experiments.

Enzyme Purification
A cell-free culture of the *Absidia* sp.d38 strain was treated with 40% saturated (NH$_4$)$_2$SO$_4$ to remove any precipitate, and further treated with 75% saturated (NH$_4$)$_2$SO$_4$ to harvest the enzyme. The crude enzyme solution was then eluted on a DEAE-cellulose DE-52 (Whatman) column (Φ2.0 cm×11 cm) and fractionated to collect different fractions, as shown in Fig. 3.

The dioscin-hydrolyzing activity of each fraction was assayed with dioscin as the substrate, and fractions 61 and 62 were found to hydrolyze dioscin effectively, indicating that these fractions contained dioscin-glycosidase.

Further purification of the above fractions was performed using vertical slab polyacrylamide gel electrophoresis: the enzyme in fractions 61 and 62 eluted by a 0.24 M KCl solution formed one single band on the gel. The enzyme band on the gel was then cut and dissolved in a 0.02 M acetate buffer (pH 5.0) to obtain the pure enzyme. The yield of the special dioscin-glycosidase after the DEAE-cellulose column separation was about 2.6%, and the specific activity of the enzyme was increased 7.8 times compared with that of the cell-free culture. To confirm the purity of the enzyme, further separation was conducted using polyacrylamide gel electrophoresis, however, the enzyme specific activity was not changed and the yield was only 0.66%, as shown in Table 1. HPLC was also used to check the purity of the dioscin-glycosidase. Only one peak appeared on the HPLC, at 15.857 min, indicating that the dioscin-glycosidase separated by the DEAE and PAGE was already a pure enzyme. This datum was omitted. Thus, since the enzyme acquired through the DEAE-cellulose column separation was already concluded to be a pure dioscin-glycosidase, it was evaluated for its molecular mass and enzymatic properties.

### Molecular Mass of New Dioscin-Glycosidase
SDS–polyacrylamide gel electrophoresis was used to estimate the molecular mass of the new dioscin-glycosidase from the *Absidia* sp.d38 strain. The purified enzyme from

<table>
<thead>
<tr>
<th>Step</th>
<th>Volume (ml)</th>
<th>Total activity (U)</th>
<th>Total protein (mg)</th>
<th>Specific activity (U/mg)</th>
<th>Purification fold (χ fold)</th>
<th>Yield (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fermentation</td>
<td>400</td>
<td>18,500</td>
<td>1246</td>
<td>14.8</td>
<td>1.0</td>
<td>100</td>
</tr>
<tr>
<td>(NH$_4$)$_2$SO$_4$ precipitation</td>
<td>40</td>
<td>11,816</td>
<td>609.1</td>
<td>19.4</td>
<td>1.3</td>
<td>64.9</td>
</tr>
<tr>
<td>DEAE-cellulose (4 times)</td>
<td>12</td>
<td>486</td>
<td>4.2</td>
<td>115.7</td>
<td>7.8</td>
<td>2.6</td>
</tr>
<tr>
<td>Electrophoresis</td>
<td>2</td>
<td>122</td>
<td>1.05</td>
<td>116.2</td>
<td>7.9</td>
<td>0.66</td>
</tr>
</tbody>
</table>
fraction 61 formed one band on the SDS–polyacrylamide gel, as shown in Fig. 4.

Standard proteins were run together with the enzyme, and the enzyme molecular weight calculated using a standard curve and plotting the log of the molecular weights of the standard proteins vs. the relative mobility of the proteins. The molecular mass of the new dioscin-glycosidase was determined to be about 55 kDa.

**Enzymatic Hydrolysis of Dioscin**

The products of the hydrolysis of dioscin by the new dioscin-glycosidase are shown in Fig. 5.

The hydrolysis products of the dioscin-glycosidase on dioscin had the same Rf values as 3-O-α-L-Rha-β-n-Glc-diosgenin and diosgenin (aglycone), and a small amount of 3-O-α-L-Rha-β-n-Glc-diosgenin, indicating that the new dioscin-glycosidase gradually hydrolyzed either 3-O-α-L-(1→4)-Rha or 3-O-α-L-(1→2)-Rha from dioscin into 3-O-α-L-Rha-β-n-Glc-diosgenin, while also rapidly hydrolyzing the other α-L-Rha from 3-O-α-β-Rha-β-n-Glc-diosgenin into the main intermediate products of 3-O-β-n-Glc-diosgenin, and then hydrolyzing these intermediate products into aglycone as the final product.

To investigate the ability of the enzyme to hydrolyze another glycoside-diosgenin, 3-O-[α-L-Rha-(1→2), α-L-Ara-(1→4)]-β-n-Glc-diosgenin was reacted with the enzyme, as shown in Fig. 5. The dioscin-glycosidase also gradually hydrolyzed the 3-O-(1→4)-α-L-arabinoside, 3-O-(1→2)-α-L-rhamnoside, and β-n-glucoside on diosgenin into low-

**Properties of New Dioscin-Glycosidase**

The effects of the pH, temperature, and reaction time on the new dioscin-glycosidase-catalyzed dioscin hydrolysis were evaluated, and the results are shown in Fig. 7.

Fig. 7A shows that the optimal pH was 5.0, are Fig. 7B shows that the optimal temperature was 40°C, and Fig. 7C shows that the optimal reaction time was 30 h.

The effects of various metal ions on the new dioscin-glycosidase were also investigated and are listed in Table 2. It appeared that the activity of the new dioscin-glycosidase was not affected by Na⁺, K⁺, and Mg²⁺ ions as high as 50 mM, but was significantly inhibited by Cu²⁺ and Hg²⁺ ions at 10 mM, and slightly affected by Ca²⁺ ions at 10 mM.

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**Fig. 5.** Hydrolysis of dioscin by new dioscin-glycosidase. 1, Standard dioscin; ①, Enzyme reaction product from dioscin; 2, Standard 3-O-[α-(1→4)-Ara, α-(1→2)-Rha]-β-n-Glc-diosgenin; ②, Enzyme reaction product from 3-O-[α-L-Rha-(1→2)-Rha]-β-n-Glc-diosgenin; 3, Standard 3-O-α-L-Ara-β-n-Glc-diosgenin; 4, Standard 3-O-β-n-Glc-diosgenin; 5, Standard diosgenin (aglycone); Substrate, 2.0 mg/ml and reacted at 40°C for 20 h; Solvent, chloroform:methanol:water =7:3:0.5 (underlayer).

**Fig. 6.** Enzyme hydrolysis products from dioscin analyzed by HPLC. 1, Dioscin; 2, 3-O-α-L-Rha-β-n-Glc-diosgenin; 3, 3-O-β-n-Glc-diosgenin; 4, diosgenin (aglycone). Enzyme, 20 U/ml; Substrate, 4.0 mg/ml dioscin, reacted at 40°C for 20 h.
Table 2. Effects of metal ions on new dioscin-glycosidase (relative activity, %).

<table>
<thead>
<tr>
<th>Metal ions</th>
<th>None</th>
<th>Na⁺</th>
<th>K⁺</th>
<th>Ca²⁺</th>
<th>Mg²⁺</th>
<th>Cu²⁺</th>
<th>Hg²⁺</th>
</tr>
</thead>
<tbody>
<tr>
<td>Concentration (mM)</td>
<td>0</td>
<td>10</td>
<td>50</td>
<td>10</td>
<td>50</td>
<td>10</td>
<td>50</td>
</tr>
<tr>
<td>Enzyme activity (%)</td>
<td>100</td>
<td>99</td>
<td>102</td>
<td>103</td>
<td>100</td>
<td>107</td>
<td>67</td>
</tr>
</tbody>
</table>

Enzyme, 10 U/ml; substrate, dioscin concentration 2.0 mg/ml; reacted at 40°C and pH 5.0 for 20 h.
Table 3. Comparison of dioscin-glycosidases.

<table>
<thead>
<tr>
<th>Enzyme</th>
<th>Enzyme source</th>
<th>M (kDa)</th>
<th>Hydrolyzing glycoside type of dioscin</th>
<th>Final enzyme products</th>
<th>Optimal temperature (°C)</th>
<th>Optimal pH</th>
</tr>
</thead>
<tbody>
<tr>
<td>Dioscin-glycosidase in this paper</td>
<td>Absidia sp.d38 stain</td>
<td>55</td>
<td>α-(1→2)-Rha</td>
<td>Aglycone</td>
<td>40</td>
<td>5.0</td>
</tr>
<tr>
<td>Glucoamylase</td>
<td>Curvularia lunata strain</td>
<td>66</td>
<td>α-(1→2)-Rha</td>
<td>3-O-Rha-Glc-diosgenin</td>
<td>50</td>
<td>4.0</td>
</tr>
<tr>
<td>Dioscin-α-1-rhamnosidase</td>
<td>Pig liver</td>
<td>47</td>
<td>α-(1→2)-Rha</td>
<td>3-O-Rha-Glc-diosgenin</td>
<td>42</td>
<td>7.0</td>
</tr>
<tr>
<td>Dioscinase</td>
<td>Microorganism sp.s00e strain</td>
<td>59</td>
<td>α-(1→4)-Rha</td>
<td>3-O-Glc-diosgenin</td>
<td>40</td>
<td>5.0</td>
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</tbody>
</table>

References


Acknowledgment

This work was supported by the Ministry of Education of Liaoning Province of P. R. China (2007T006) and National Science Foundation of China (NSFC).