Purification and Some Properties of Glycyrrhizinic Acid Hydrolase from Aspergillus niger GRM3

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We selected Aspergillus niger GRM3 because it secreted a novel enzyme that hydrolyzed glycyrrhizinic acid. The enzyme was purified to the electrophoretically homogeneous state and an activity more than 890-fold that of culture broth. The molecular weight of the enzyme was estimated to be 150,000 by gel filtration and it was most active over the range of pH 4.1 to 4.5. The enzyme hydrolyzed glycyrrhizinic acid to produce glycyrrhetinic acid and 6-glucuronyl-β-1,2-d-glucuronic acid. The hydrolytic action of this enzyme was different from those of β-glucuronidases so far reported.

Roots of Glycyrrhiza spp., commonly called licorice, contain the pentacyclic triterpene saponin glycyrrhizinic acid. Glycyrrhizinic acid consists of the aglycone β-glycyrrhetinic acid and two molecules of d-glucuronic acid attached to the C-3 atom of the aglycon moiety.1,2) Licorice is presently recognized for its sweet taste,3,4) and aglycone glycyrrhetinic acid is also used for its pronounced anti-ulcer5–7) and antiinflammatory properties.8–11) Glycyrrhetinic acid is now produced from glycyrrhizinic acid by acid hydrolysis. However, it is difficult to obtain glycyrrhetinic acid in a good yield by this method, because many by-products are also produced.

To find an enzyme which efficiently catalyzes the hydrolysis of glycyrrhizinic acid, we screened various microorganisms. A unique enzyme was prepared from a strain of Aspergillus niger GRM3 and the action of this enzyme (glycyrrhizinic acid hydrolase) was different from those of the β-glucuronidases12–14) so far reported.

MATERIALS AND METHODS

Materials. β-Glucuronidase from a marine mollusc was purchased from P-L Biochemicals Inc. Monoammonium glycyrrhizinate was recrystallized four times from 85% methanol. α-Glucosyl glycyrrhizinic acid was prepared from glycyrrhizinic acid and starch by cyclomaltodextrin glucanotransferase from Bacillus stearothermophilus.15) All other chemicals used were of reagent grade.

Cultivation. Aspergillus niger GRM3 was cultivated with shaking in a medium (pH 5.8) containing 2.5% corn steep liquor, 2.5% soybean flour-alkali extracts, 1% sophora flowers and 0.5% CaCO3 at 27°C for 4 days. After cultivation, the culture broths were obtained by centrifugation and the supernatants were used as the enzyme solutions.

Assay of hydrolytic activity of glycyrrhizinic acid hydrolase. A half milliliter of the enzyme solution was incubated with 0.5 ml of monoammonium glycyrrhizinate in 1 M acetate buffer (pH 5.0) at 40°C. At an appropriate time, 4 ml of ethyl acetate was added to the reaction solution and then was vigorously stirred. After centrifugation, the amounts of glycyrrhetinic acid in the layer of ethyl acetate were measured at 265 nm using a Shimadzu UV-210A spectrophotometer. One unit of the enzyme activity was defined as the amount of enzyme which produced 1 μmol of glycyrrhetinic acid per min from glycyrrhizinic acid.
Measurement of protein. Protein was measured spectrophotometrically by the absorbance at 280 nm using a Shimadzu UV-210A spectrophotometer.

Polyacrylamide gel electrophoresis. Disc electrophoresis was done by the method of Davis and Nagai in a column of 7.5% polyacrylamide gel with a pH 9.4 buffer system. Electrophoresis was done at a current of 3 mA per column for 2.5 hr.

Isoelectric focusing. Isoelectric focusing was done by the method of Matsuo in a 110 ml column using carrier ampholites (pH 3–10, LKB Product AB, Sweden) for 48 hr at 300 V, 4°C.

Thin layer chromatography. Thin layer chromatography (TLC) was done with a HPTLC plate (Kiselgel 60 F254) purchased from Merck Japan Ltd., by the ascending method, using n-butanol-3N ammonia water-ethanol (5:2:1) for the identification of aglycone, and benzene-acetic acid-methanol (1:1:3) for the identification of sugar components. The products on the TLC plates were detected with 50% sulfuric acid.

Carbon-13 nuclear magnetic resonance ($^{13}$C-NMR). The $^{13}$C-NMR spectra were taken on a JEOL FX-100 spectrometer (25.00 MHz) in D$_2$O with dioxane as an internal standard.

RESULTS

Purification of glycyrrhizinic acid hydrolase

Aspergillus niger GRM3 was cultivated with shaking at 27°C for 4 days. After centrifugation, the supernatant was used as the starting material for the purification.

Step. 1. Ammonium sulfate fractionation. To the supernatant (9000 ml), solid ammonium sulfate was added up to 0.6 saturation. The resulting precipitate was collected by filtration and dissolved in a small amount of 0.02M acetate buffer, pH 5.0. The solution was dialyzed against the same buffer.

Step. 2. SP-Sephadex C-50 column chromatography. The dialyzed solution (750 ml) was put on a SP-Sephadex C-50 column (3 x 66 cm) which had been equilibrated with 0.02M acetate buffer, pH 5.0. The column was washed with the same buffer and glycyrrhizinic acid hydrolase was eluted by a linear gradient of ionic strength from 0 to 0.7 NaCl. The active fractions were combined and dialyzed against 0.02M acetate buffer (pH 5.0) containing 0.1M NaCl.

Step. 3. Sephadex G-200 gel filtration. The dialyzed solution was concentrated to 5 ml by dialyzing against polyethylene glycol (#20,000). The concentrated solution was put on a Sephadex G-200 column (2 x 100 cm) which had been equilibrated with 0.02M acetate buffer, pH 5.0, containing 0.1M NaCl. Elution was done with the same buffer.

Step. 4. Isoelectric focusing. The active fractions were combined and concentrated to 4 ml by dialyzing against polyethylene glycol (#20,000). The concentrated solution was used for electrophoresis. An electrophoretic profile is shown in Fig. 1. The active fractions were combined and used as the purified enzyme preparation. This preparation was purified about 890-fold from the culture broth and the specific activity of glycyrrhizinic acid hydrolase was 8.9 units/Abs. at 280 nm. This purified enzyme was used in the following experiments. The results of the purification procedures are summarized in Table I.

Homogeneity of the purified enzyme preparation

Homogeneity of the purified enzyme preparation was tested by disc electrophoresis on polyacrylamide gels. From the result shown in Fig. 2, the enzyme preparation was considered to be homogeneous.

Effects of temperature and pH on stability and activity

Glycyrrhizinic acid hydrolase was stable at temperatures below 45°C and stable over the range of pH 5.0 to 6.5, as shown in Fig. 3. The optimum temperature of the enzyme was 45°C. The enzyme was most active over the range of pH 4.1 to 4.5, as shown in Fig. 4.

Molecular weight

The molecular weight of glycyrrhizinic acid hydrolase was estimated by the gel filtration method. A column of Sephadex G-200 (2 x 100 cm) eluted with 0.02M acetate buffer, pH 5.0, containing 0.1M NaCl, was used. The molecular weight of the enzyme was estimated.
Glycyrrhizinic Acid Hydrolase from A. niger GRM3

Fig. 1. Isoelectric Focusing of Glycyrrhizinic Acid Hydrolase from A. niger GRM3.
The experimental conditions are described in the text. ●, hydrolytic activity of glycyrrhizinic acid hydrolase; ○, absorbance at 280 nm; ---, pH.

Table I. Purification of Glycyrrhizinic Acid Hydrolase from Aspergillus niger GRM3

<table>
<thead>
<tr>
<th>Purification steps</th>
<th>Protein $A_{280}$</th>
<th>Total activity (U)</th>
<th>Specific activity (U/$A_{280}$)</th>
<th>Yield (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Supernatant fluid of broth</td>
<td>173,000</td>
<td>1,730</td>
<td>0.01</td>
<td>100</td>
</tr>
<tr>
<td>Precipitates with (NH$_4$)$_2$SO$_4$</td>
<td>12,420</td>
<td>1,490</td>
<td>0.12</td>
<td>86</td>
</tr>
<tr>
<td>Eluate from SP-Sephadex C-50</td>
<td>845</td>
<td>490</td>
<td>0.58</td>
<td>29</td>
</tr>
<tr>
<td>Eluate from Sephadex G-200</td>
<td>50</td>
<td>230</td>
<td>4.6</td>
<td>13</td>
</tr>
<tr>
<td>Preparation from isoelectric focusing</td>
<td>7.3</td>
<td>65</td>
<td>8.9</td>
<td>4</td>
</tr>
</tbody>
</table>

to be 150,000 by gel filtration using ferritin, catalase, aldolase, ovalbumin, and bovine serum albumin as standard proteins.

Action of glycyrrhizinic acid hydrolase
The action of the enzyme on various compounds is shown in Table II. Glycyrrhizinic acid was the best substrate for this enzyme, and $p$-nitrophenyl-$\beta$-$D$-glucuronide and phenolphthalein-$\beta$-$D$-glucuronide which were the substrate for $\beta$-glucuronidase, were not good substrates.

The actions of glycyrrhizinic acid hydrolase and $\beta$-glucuronidase on glycyrrhizinic acid are shown in Fig. 5. $\beta$-Glucuronidase produced the monoglucuronide of glycyrrhetinic acid and glucuronic acid from glycyrrhizinic acid. On the other hand, glycyrrhizinic acid

Fig. 2. Polyacrylamide Gel Electrophoresis of Glycyrrhizinic Acid Hydrolase from A. niger GRM3.
The purified enzyme (100 $\mu$g) was electrophoresed at pH 9.4 as described in the text.
Fig. 3. Effects of pH and Temperature on Stability of Glycyrrhizinic Acid Hydrolase. Conditions: (a) 40°C, 1 hr (pre-incubation); (b) pH 5.0, 30 min (pre-incubation).

Fig. 4. Effects of pH and Temperature on Activity of Glycyrrhizinic Acid Hydrolase. Conditions: (a) 40°C, 30 min (reaction time); (b) pH 5.0, 30 min (reaction time).

Fig. 5. Actions of β-Glucuronidase and Glycyrrhizinic Acid Hydrolase on Glycyrrhizinic Acid. A, glycyrrhizinic acid; B, monoglucuronide of glycyrrhetinic acid; C, glycyrrhetinic acid; D, glucuronic acid. Solvent system: benzene–acetic acid–methanol (1:1:3).
Hydrolase produced glycyrrhetinic acid and an unknown product (UP) (Fig. 5), although the monoglucuronide of glycyrrhetinic acid and glucuronic acid were not produced.

To obtain large amounts of UP, it was prepared from the reaction solution by partitioning between water and chloroform. Consequently, aglycone glycyrrhetinic acid was extracted by chloroform and UP remained in the water layer. As shown in Fig. 6, it was recognized that glucuronic acid was produced from UP by β-glucuronidase digestion. After recrystallization of UP by 50% methanol, its structure was analyzed by 13C-NMR. The 13C-NMR spectrum is shown in Fig. 7. The peaks (81.4, 81.8, 103.2, and 104.6 ppm) provided the definitive evidence for the existence of the β-1,2-glucuronide bond in the structure of UP.

**Table II. Actions of Glycyrrhizinic Acid Hydrolase on Various Compounds**

<table>
<thead>
<tr>
<th>Compounds</th>
<th>Hydrolytic activity</th>
</tr>
</thead>
<tbody>
<tr>
<td>Glycyrrhizinic acid</td>
<td>++</td>
</tr>
<tr>
<td>α-Glucosyl glycyrrhizinic acid</td>
<td>+</td>
</tr>
<tr>
<td>p-Nitrophenyl-β-D-glucuronide</td>
<td>-</td>
</tr>
<tr>
<td>Phenolphthalein-β-D-glucuronide</td>
<td>-</td>
</tr>
</tbody>
</table>

++, greatly hydrolyzed; +, hydrolyzed; -, not hydrolyzed.

![Fig. 6. Action of β-Glucuronidase on UP.](image)

Twenty microliters of β-glucuronidase (11 mg/ml) were added to 0.2 ml of 1% UP in 0.02 M acetate buffer (pH 5.0). At appropriate times, the reaction mixtures were chromatographed on a TLC plate, using the solvent system of benzene, acetic acid, and methanol (1:1:3). UP, unknown product; G, glucuronic acid.

![Fig. 7. 13C-NMR Spectrum of UP Produced from Glycyrrhizinic Acid by Glycyrrhizinic Acid Hydrolase.](image)

The chemical shifts were given in δ (ppm) with dioxane as an internal standard.
DISCUSSION

Previously described $\beta$-glucuronidases from various animals and plants catalyzed the hydrolysis of glycosides having glucuronic acid by cleaving the glucuronic acid unit, and then producing glucuronic acid and other products.\(^{19}\)

Our purified glycyrrhizinic acid hydrolase produced glycyrrhetinic acid and the unknown product (UP) from glycyrrhizinic acid, and glucuronic acid was produced from UP by $\beta$-glucuronidase. Furthermore, it was found that a $\beta$-1,2-glucuronic acid bond was present in the structure of UP from the $^{13}$C-NMR spectrum. From these results, it was considered that UP was D-glucuronyl-$\beta$-1,2-D-glucuronic acid.

Therefore, this enzyme had a novel action. Furthermore, the application of this enzyme to the structure analyses of glycosides having glucuronide bonds should be interesting, since this enzyme produced $\alpha$-glucosyl glucuronosiose and glycyrrhetinic acid from $\alpha$-glucosyl glycyrrhizinic acid.

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