Isolation and Some Properties of the Enzyme That Transforms Eremofortin C to PR Toxin

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PR toxin and eremofortin C are secondary metabolites of Penicillium roqueforti. The chemical structures of these two compounds are closely related to each other and differ only by an aldehyde and an alcohol group at the C-12 position. In an effort to better understand the biosynthesis of PR toxin, we discovered the enzyme of \( P. \) \( \text{roqueforti} \) that is responsible for the transformation of eremofortin C to PR toxin. The maximum activity of the enzyme in the culture medium was found to occur on day 13, which corresponded to the maximal production of PR toxin in the medium. The enzyme was isolated and purified from the culture medium and the mycelium of the fungus, respectively, through a procedure involving ammonium sulfate fractionation and DEAE-cellulose chromatography. The specific activity increased 20- and 8-fold, respectively, and the yield was 33.3 and 21.6\%, respectively, for the enzyme from the medium and mycelium. The optimal pH for the enzyme reaction was ca. pH 5.6. The enzyme reaction was temperature dependent. The rates followed a linear time course when it catalyzed the transformation at 30°C and decayed with time when reacted at higher temperatures. At 100°C, the enzyme activity was completely lost. The \( K_m \) and \( V_{max} \) of the enzyme as determined at 30°C were 0.02 mM and 4.0 \( \mu \)mol/min per mg, respectively. The molecular weight of the enzyme was estimated by gel filtration on a high-pressure liquid chromatography 1-250 protein column to be ca. 40,000.

MATERIALS AND METHODS

Preparation of EC and PR toxin standards. PR toxin and EC were isolated from the culture medium of \( P. \) \( \text{roqueforti} \) by chloroform extraction and further purified by methods described previously (7, 17). Crystalline EC and PR toxin were prepared as described by Moreau et al. (7) and Wei et al. (17), respectively, and dissolved in chloroform as standards.

HPLC. PR toxin and EC were analyzed by HPLC, with chloroform as the solvent, at a flow rate of 2 ml/min as described by Moreau et al. (9). HPLC analyses were carried out with a Waters ALC/GPC 204 instrument equipped with a U6K universal injector, an M-6000 A pump, a \( \mu \)Porasil column (3.9 mm by 30 cm), and a model 440 absorbance detector with a 254-nm filter. Chromatograms were recorded on an Omnicrisc 10-in (ca. 25.4-cm) recorder (Houston Instruments, Div. Bausch & Lomb, Inc., Austin, Tex.). A sensitivity of 0.1 absorbance units, full scale, was used. The retention times of PR toxin (2 min) and EC (6 min) were verified with EC and PR toxin standards. The amounts of PR toxin and EC in the sample were quantitated by the measurement of the peak height of each compound in the chromatogram.

Purification of the transforming enzyme. The ATCC 10110 strain of \( P. \) \( \text{roqueforti} \) was grown in a culture medium containing 2% yeast extract and 15% sucrose which had been autoclaved at 121°C for 15 min and incubated at 25°C in the dark as described previously (17). On day 13, the culture medium was harvested and filtered in a Buchner funnel through a Whatman no. 3 filter paper. A small portion of the filtrate was dialyzed against 10 mM Tris-hydrochloride buffer (pH 8.0) and assayed for enzyme activity. The rest of the filtrate (1.4 \( \times \) 10\(^3\) ml) was then slowly mixed with ammonium sulfate to 70% by constant stirring at 4°C. The solution was allowed to stand in ice water for 1 to 2 h. The precipitate obtained by centrifugation at 10,000 \( \times \) g for 20 min was dissolved in 180 ml of 10 mM Tris-hydrochloride buffer (pH
The dialysate was subjected to a cellulose column containing 40 ml of DEAE-cellulose (2.5 by 9.0 cm) which had been equilibrated with 10 mM Tris-hydrochloride buffer (pH 8.0). Dialysis was carried out at 30°C for 10 min (B) and 30 min (C) by the addition of 1 ml of culture medium dialysate. The reaction proceeded as described in the text.

FIG. 1. HPLC chromatograms showing the transformation of EC to PR toxin by the culture medium dialysate. One microgram of PR toxin and 3 μg of EC were mixed and used as standards (A). Transformation was carried out at 30°C for 10 min (B) and 30 min (C) by the addition of 1 ml of culture medium dialysate. The reaction proceeded as described in the text.

8.0). The solution was desalted by dialysis overnight against 1.5 liters of the same buffer at 4°C with one change of the buffer. The dialysate was applied onto a DEAE-cellulose column (3.5 by 9.0 cm) which had been equilibrated with 10 mM Tris-hydrochloride buffer (pH 8.0). After washing with 2 bed volumes of the column buffer, the column was eluted with a stepwise gradient of 140 ml each of 50, 100, 200, and 500 mM NaCl in 10 mM Tris-hydrochloride buffer (pH 8.0). The fractions containing the enzyme activity were pooled and further purified by gel filtration on a Waters HPLC I-250 protein column in case higher purity was needed. On the other hand, 250 g of the mycelium was washed thrice with 0.85% saline water and frozen at −70°C overnight. The frozen mycelium was disrupted by grinding with the aid of SiO2 powder and suspended in 550 ml of 10 mM Tris-hydrochloride buffer (pH 8.0). The suspension was centrifuged at 1,000 × g for 15 min, and the pellet was discarded. A small portion of the supernatant was dialyzed and assayed for enzyme activity, and the rest was subjected to 70% ammonium sulfate precipitation. The pellet obtained from centrifugation was dissolved in 60 ml of 10 mM Tris-hydrochloride buffer (pH 8.0) and dialyzed overnight against the same buffer. The dialysate was chromatographed onto a DEAE-cellulose column as described above.

Assay of transforming enzyme activity. The transformation activity of the enzyme was determined by HPLC analysis of the amount of EC that was converted to PR toxin by a suitable amount of the enzyme. The assay mixture, in a total volume of 9.5 ml, contained 0.25 M sucrose, 0.5 mM MgCl2, and 10 mM phosphate buffer (pH 5.6). After incubation at 30°C for 5 min, the assay mixture was supplemented with 3.1 μmol of EC (dissolved in 45 μl of methanol) and 0.5 ml of the enzyme solution. The reaction proceeded at 30°C for 10 min and was then terminated by the addition of 10 ml of cold chloroform. After thorough mixing, the chloroform layer was collected and evaporated at 45°C under a gentle stream of N2 gas. The sample was then dissolved in 1 ml of chloroform, and a portion of 10 μl was injected into an HPLC μPorasil column for the measurement of the amount of PR toxin that was converted from EC by the enzyme. A control experiment was performed by the same procedure, except that no enzyme was added to the assay mixture, and another control was done with the same assay mixture containing an identical amount of the enzyme but without the addition of EC. These two controls were made to ensure that the PR toxin detected in the assay mixture after the enzyme reaction was indeed converted from EC. One unit of the enzyme activity was defined as the amount of enzyme that catalyzed the transformation of 1 μmol of EC to PR toxin per min under the assay condition. Specific activity was defined as the amount of enzyme activity per milligram of protein.

Effects of pH and temperature on enzyme activity. To determine the optimal pH for the enzyme reaction, we assayed the transformation activity of the purified enzyme at 30°C in the assay mixture made at different pHs by using the following buffer systems: potassium chloride-hydrochloric acid, pH 2.0; sodium citrate, pH 3.4; sodium phosphate, pH 5.6 and 7.0; and Tris-hydrochloride, pH 8.0 and 10.0. The other assay conditions and procedures were the same as described above.

The effect of temperature on the enzyme activity was studied by the measurement of the transformation activity of the purified enzyme at 30, 40, 60, 70, and 100°C. The enzyme reaction at each temperature proceeded for 2, 5, 10, and 20 min.

Kinetic properties of the enzyme. The kinetic properties of the enzyme were studied by the determination of the enzyme activity at 30°C with a series of different concentrations of the substrate in the assay mixture as described above.

Molecular weight determination. The enzyme as purified from the DEAE-cellulose column was injected into an HPLC I-250 protein column for the analysis of purity. The peak fraction that contained the enzyme activity was collected and reinjected into the same column for molecular weight determination. The I-250 column was equilibrated with 50 mM sodium phosphate buffer (pH 6.8) and eluted with the same buffer at a flow rate of 1.0 ml/min after the injection of sample. Human serum albumin (67,000), ovalbumin (43,000), chymotrypsinogen A (25,700), and cytochrome c (12,400) were used as standards. Protein concentration was determined by the Lowry method (6), with bovine serum albumin as the standard (absorbance at 280 nm = 6.6 for 1% solution).

RESULTS

When EC was incubated in the buffer solution containing the dialysate of the culture medium that had been grown with P. roqueforti, a new product, PR toxin, gradually appeared, and the amount of EC decreased (Fig. 1). These results indicate that the culture medium contains the enzyme system that is responsible for the transformation of EC to PR toxin. A similar finding was observed for the crude extract of the mycelium of P. roqueforti. When we examined the...
activity profile of the enzyme in the culture medium during the growth period of the fungus, we found the maximum activity on day 13 of the culture (Fig. 2). Thus, on this day we harvested both the culture medium and the mycelium for the isolation of the transforming enzyme. The purification procedures are summarized in Tables 1 and 2. At 70% saturation, ammonium sulfate precipitated ca. 77% of the enzyme activity from the culture medium dialysate and crude extract of the mycelium, respectively. After dialysis of the precipitate, the enzyme solution was further purified by DEAE-cellulose column chromatography. The purity of the enzymes from the two sources were increased 20- and 8-fold, and the yields were 33.3 and 21.6%, respectively. The elution profile of the DEAE-cellulose column for the purification of the enzyme from the medium was similar to that for the mycelium (Fig. 3). In both cases, the enzyme activity started to appear and was completely eluted from the column at salt concentration of 100 mM NaCl.

The optimal pH for the transformation reaction of the enzyme was studied over a pH range of 2.0 to 10.0. The reaction pH profile of the enzyme from the culture medium was found to be identical to that of the enzyme from the mycelium. The optimal pH was found to be at pH 5.6 (Fig. 4). A sharp decrease in the enzyme activity was observed at pH 2.0 and 10.0. When the enzyme was assayed with various buffer systems, including phosphate, citrate, acetate, and succinate buffers (all at pH 5.6), we found no significant difference in the transforming activity of the enzyme. Furthermore, we found that the enzyme activity was essentially the same when assayed at buffer concentrations of 10 to 200 mM. These findings indicate that the enzyme reaction is not affected by either the type or the ionic strength of the buffers.

To determine the optimal reaction temperature for the enzyme reaction, we measured the enzyme activity at pH 5.6 over the temperature range of 30 to 100°C. Figure 5 shows that between 30 and 60°C the reaction followed a linear time course for 10 min; only at 30°C could linearity be maintained for 20 min. The reaction curve was curvilinear at 40°C, but bent sharply at 60°C after 10 min of reaction. Only a very small fraction of the activity remained at 70°C, and the enzyme was completely denatured at 100°C.

Kinetic properties of the enzyme were studied by assaying the activity at various concentrations of the substrate. The results showed that the enzyme was gradually saturated at substrate concentrations above 0.03 mM and completely saturated at 0.3 mM (Fig. 6). The Lineweaver-Burk double-reciprocal plot of the data revealed that the Km and Vmax of the enzyme were 0.02 mM and 4.0 μmol/min per mg, respectively.

When the purified enzyme obtained from the DEAE-cellulose column was subjected to gel filtration on an HPLC I-250 protein column, three major peaks were observed in the elution profile (Fig. 7, peaks b to d). Ca. 90% of the enzyme activity was located in only one peak (peak b). When the peak b fraction was injected into the I-250 protein column, we found in the elution profile that peak b was still contaminated with some peak c proteins (Fig. 7, lower trace). Since only the peak b fraction showed transformation

FIG. 2. Time course of the production of the transforming enzyme by P. roqueforti. Ten milliliters of the culture medium was pipetted without damaging the mycelium at the indicated time. Transformation was carried out by the addition of 1.0 ml of culture medium dialysate to 9.0 ml of the assay mixture, and the activity was assayed as described in the text.

FIG. 3. Elution profile of the DEAE-cellulose column. Thirty milliliters of the enzyme solution (0.4 mg/ml) in 10 mM Tris-hydrochloride buffer (pH 8.0) was applied onto a DEAE-cellulose column (3.5 by 9.0 cm) which had been equilibrated with the same buffer. After washing with 2 bed volumes of the buffer, the column was eluted with the same buffer containing stepwise gradients from 0.05 to 0.5 M NaCl. The flow rate was 2 ml/min, and the volume for each fraction was 10 ml. The absorbance at 280 nm (solid line) and enzyme activity (dotted line) of each fraction were measured to locate the enzyme.

### TABLE 1. Purification of the enzyme from the culture medium of P. roqueforti

<table>
<thead>
<tr>
<th>Fraction</th>
<th>Vol (ml)</th>
<th>Amt (mg) of total protein</th>
<th>Total activity (U)</th>
<th>Sp act (U/mg)</th>
<th>% Yield</th>
<th>Purification (fold)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Medium dialysate</td>
<td>1,400</td>
<td>630</td>
<td>119.7</td>
<td>0.2</td>
<td>100</td>
<td>1</td>
</tr>
<tr>
<td>70% (NH₄)₂SO₄</td>
<td>180</td>
<td>72</td>
<td>92.3</td>
<td>1.3</td>
<td>77.1</td>
<td>6.5</td>
</tr>
<tr>
<td>DEAE-cellulose</td>
<td>240</td>
<td>10.5</td>
<td>40.5</td>
<td>3.9</td>
<td>33.8</td>
<td>19.5</td>
</tr>
</tbody>
</table>

The enzyme activity (absorbed at 280 nm) of the enzyme was measured as described in the text. The enzyme activity was determined as described in the text.

### TABLE 2. Purification of the enzyme from the mycelium of P. roqueforti

<table>
<thead>
<tr>
<th>Fraction</th>
<th>Vol (ml)</th>
<th>Amt (mg) of total protein</th>
<th>Total activity (U)</th>
<th>Sp act (U/mg)</th>
<th>% Yield</th>
<th>Purification (fold)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mycelium extract</td>
<td>550</td>
<td>1,100</td>
<td>53.7</td>
<td>0.05</td>
<td>100</td>
<td>1</td>
</tr>
<tr>
<td>70% (NH₄)₂SO₄</td>
<td>60</td>
<td>366</td>
<td>29.2</td>
<td>0.08</td>
<td>54.3</td>
<td>1.6</td>
</tr>
<tr>
<td>DEAE-cellulose</td>
<td>180</td>
<td>28</td>
<td>11.6</td>
<td>0.41</td>
<td>21.6</td>
<td>8.2</td>
</tr>
</tbody>
</table>
Since the discovery and isolation of PR toxin (17), it has been of great interest to mycotoxin investigators to elucidate the biosynthetic pathway of the toxin and other related secondary metabolites (7-9, 12). In an earlier time course study, we found that there is an intimate relationship between the production of EC and PR toxin (16). The later studies by Moreau and co-workers (7-9) have further shown that EC is produced by P. roqueforti earlier than PR toxin and that the decrease in the amount of EC in the culture medium is always associated with an increase in PR toxin production. These findings, together with the other evidence that the two compounds bear very similar chemical structures (7, 15) and are easily transformed between each other (7), have lead Moreau et al. (8) to propose that EC is the direct precursor of PR toxin in the hypothetical biosynthetic pathway for P. roqueforti metabolites. These authors have further proposed that the interconversion between EC and PR toxin is catalyzed by an alcohol dehydrogenase. In an effort to better understand the transformation between the two compounds by P. roqueforti, we discovered and purified the enzyme from the culture medium and the mycelium of the fungus. As shown in Fig. 1, the culture medium that had been grown with P. roqueforti contained the enzyme that catalyzed a time-dependent transformation of EC to PR toxin. The time course of the production of the enzyme (Fig. 2) correlated well with that of PR toxin production in the culture medium (8, 14). The crude extract of the mycelium also exhibited the same enzyme activity. The control that lacked the enzyme from either source failed to show any transformation of EC to PR toxin. The dialysate of the culture medium was found to contain no EC or PR toxin. These observations clearly indicate that there exists in the culture medium an enzyme that is responsible for the transformation of EC to PR toxin in the reaction mixture.
Moreover, we found that the enzyme started to appear in the culture medium on day 7, reached a maximum on day 13, and gradually decreased thereafter (Fig. 2).

We then purified the enzyme from the culture medium and the mycelium by 70% ammonium sulfate fractionation and DEAE-cellulose column chromatography, respectively (Tables 1 and 2). Before purification, the culture medium contained ca. 70% of the total enzyme activity of P. roqueforti and exhibited much higher specific activity (ca. 40-fold) as compared with that of the mycelium. This observation and the report of Moreau et al. (8) that EC and PR toxin levels in the mycelium are very low suggest that the enzyme is mostly secreted into the medium from P. roqueforti and catalyzed most, if not all, transformations of EC to PR toxin in the medium. On the other hand, since there were fewer proteins in the culture medium than in the mycelium, the specific activity of the enzyme from the medium was always much higher either before or after purification.

The optimal pH for the enzyme reaction was ca. 5.6 (Fig. 4), which was similar to the pH value of the culture medium when the peak amount of PR toxin occurred (8, 14). Ca. 80% of the enzyme activity remained at pH 3.4 and 8.0, but the activity decreased significantly at the extremes of pH 2.0 and 10.0. These results indicate that the production of PR toxin is highly dependent on the pH of the environment in which the fungus is grown. However, the transforming activity of the enzyme is not sensitive to either the ionic strength or the type of the buffer systems used for the assay. On the other hand, we found that the transformation activity of the enzyme was temperature dependent (Fig. 5). At 30°C, the enzyme reaction followed a linear time course, but the activity decayed with time when assayed at higher temperatures.

The enzyme as purified from a DEAE-cellulose column was found to be still contaminated with other proteins. There were three major protein peaks when the enzyme was analyzed on an HPLC 1-250 protein column, but only one showed significant transformation activity (Fig. 7). The molecular weight of this major peak gave an apparent molecular weight of ca. 40,000. Since gel filtration of proteins on the I-250 protein column and the like has been pointed out to be influenced by factors other than size (4), the real molecular weight of the enzyme should be reexamined by other methods.

Since the transformation of EC to PR toxin requires the removal of two hydrogen atoms at position C-12 of the EC molecule (Fig. 8), the transforming enzyme must be either an alcohol dehydrogenase or an oxidase. Moreau et al. (8) proposed that the transforming enzyme is an alcohol dehydrogenase. However, in this study, we found that the enzyme does not require NAD⁺ or NADP⁺ as the cofactor to catalyze the transformation. By using the coupling assay in the presence of peroxidase and 4-aminoantipyrine plus phenol, we were able to determine the oxidase activity of the enzyme (1). The oxidase activity was found to be dose dependent and correspond very well with the transforming activity of the enzyme (data not shown). In addition, the enzyme was found to be mostly secreted into the medium in a time-dependent manner (Fig. 2). It has been known for some time that some species of Penicillium are able to secrete oxidizing enzymes from the mycelium of the fungus to the culture medium (5). This information, together with our findings, suggests that the transforming enzyme is an oxidase. More detailed biochemical and biophysical properties are currently under investigation in our laboratory.

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LITERATURE CITED