17. Homogeneous Hyoscyamine 6β-Hydroxylase from Cultured Roots of Hyoscyamus niger

By Yasuyuki Yamada, Souichi Okabe, and Takashi Hashimoto

Research Center for Cell and Tissue Culture,
Faculty of Agriculture, Kyoto University

(Communicated by Hisateru Mitsuda, M. J. A., April 12, 1990)

Abstract. Hyoscyamine 6β-hydroxylase (EC 1.14.11.11), the first enzyme in the biosynthetic pathway leading from hyoscyamine to scopolamine, was purified to homogeneity from cultured roots of Hyoscyamus niger. The enzyme is a monomer of 38 kDa with pI 4.6. Its amino acid composition is also reported.

A key step in the biosynthesis of scopolamine in several solanaceous plants involves the 6β-hydroxylation of [S]-hyoscyamine. The enzyme responsible for this reaction is hyoscyamine 6β-hydroxylase (EC 1.14.11.11), a 2-oxoglutarate-dependent dioxygenase. This hydroxylase is abundant in the roots of alkaloid-producing plants and is expressed in a tissue- and cell-specific manner (unpublished results). We have been able to highly purify this enzyme from cultured roots of Hyoscyamus niger and to characterize its substrate specificities. An adequate amount of the homogeneous hydroxylase, however, is necessary for the production of antibodies and for amino acid sequencing. We here report further purification of the hyoscyamine 6β-hydroxylase from cultured H. niger roots and several of its physical properties.

Root cultures of H. niger L. were initiated and subcultured as described elsewhere. Prior to the experiments, the cultures were transferred to 300-ml flasks containing 75 ml of auxin-free B5 medium and cultured for seven days. These root cultures were harvested on a suction filter then frozen with liquid nitrogen and homogenized in a Waring blender. The frozen homogenate was kept at -20°C until use. Subsequent procedures were done at 4°C. The cell homogenate (510 g) was suspended in 950 ml of K-phosphate buffer (100 mM, pH 7.5) containing 0.25 M sucrose and 3 mM dithiothreitol. This suspension was ground thoroughly with sea sand in a mortar then mixed with 10% (w/v) insoluble polyvinylpyrrolidone (“Polyclar AT”; Kasei-hin Shyouji Co., Osaka), after which the mixture was passed through a composite cheesecloth-Miracloth (Calbiochem)-cheesecloth filter. The filtrate was centrifuged at 11,000 g for 30 min. The hydroxylase in the crude extract was precipitated between 58 and 85% saturation by (NH₄)₂SO₄. The precipitate obtained after centrifugation at 11,000 g for 30 min was dissolved in 43 ml of 40 mM Tris-HCl buffer (pH 7.8) containing 0.25 M sucrose, 2 mM dithiothreitol and 30% (v/v) glycerol (buffer A), the buffer having been adjusted to 20% saturation with solid (NH₄)₂SO₄.

This enzyme solution was loaded on a Butyl-Toyopearl column (2.6×24 cm; TOSOH, Tokyo) that had been equilibrated with buffer A containing (NH₄)₂SO₄ at 20% saturation. The column was treated in steps with buffer (200-ml portions) at 20, 10 and 0% saturation with (NH₄)₂SO₄. Most of the active fractions were eluted at 0% saturation with (NH₄)₂SO₄. These active fractions were combined and passed through a Sephadex G-25 M column (2.8×41 cm; Pharmacia) that had
been equilibrated with buffer A containing 50 mM NaCl, the enzyme fraction being eluted with the same buffer. This enzyme fraction was loaded on a DEAE-Toyopearl 650 M column (2.6×25 cm; TOSOH, Tokyo) that had been equilibrated with buffer A containing 50 mM NaCl, and the column washed with 150 ml of the same buffer. The enzyme was eluted with a linear gradient of 50 to 200 mM NaCl (230-ml portions) dissolved in buffer A, at a flow rate of 1 ml/min. The active fractions were pooled and concentrated with an Amicon YM-10 ultrafiltration membrane (Amicon Co.), then solid (NH₄)₂SO₄ was added to a final saturation of 20%.

The concentrated enzyme solution was loaded on a Phenyl-Superose HR 10/10 column (Pharmacia) that had been equilibrated with buffer A containing (NH₄)₂SO₄ at 20% saturation, and the column washed with 7.5 ml of the same buffer. The enzyme was eluted at a flow rate of 0.2 ml/min with a linear gradient of 20 to 0% saturation of (NH₄)₂SO₄ dissolved in buffer A (80 ml). The active fractions were pooled, concentrated by ultrafiltration as described above, then desalted by passing the concentrate through a PD-10 column (Pharmacia). The desalted enzyme was loaded on a hydroxyapatite A7610 column (Mitsui Toatsu Chemicals, Tokyo) that had been equilibrated with buffer A containing 5 mM K-phosphate (pH 6.5), and the column washed with 9 ml of the same buffer. The enzyme was eluted with a linear gradient of 5 to 200 mM K-phosphate in buffer (A 26 ml) at a flow rate of 0.15 ml/min. The active fractions obtained were pooled and stored at −20°C.

Hyoscyamine 6β-hydroxylase activity was assayed by measuring the formation of 6β-hydroxyhyoscyamine with GLC as described elsewhere.²) Protein concentrations were determined by the method of Bradford.⁶⁻

### Table I. Purification of hyoscyamine 6β-hydroxylase from cultured *H. niger* roots. *Hyoscyamus* roots were cultured and processed as described in the text. The enzyme preparation from each purification step was incubated at 30°C for 1 h in the standard assay mixture.

<table>
<thead>
<tr>
<th>Purification step</th>
<th>Total protein (mg)</th>
<th>Total activity (pkat)</th>
<th>Specific activity (pkat/mg)</th>
<th>Yield (%)</th>
<th>Purification (-fold)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Crude extract</td>
<td>1,321</td>
<td>11,371</td>
<td>8.61</td>
<td>100</td>
<td>1.0</td>
</tr>
<tr>
<td>58-85% (NH₄)₂SO₄</td>
<td>166</td>
<td>13,311</td>
<td>80.1</td>
<td>117</td>
<td>9.3</td>
</tr>
<tr>
<td>Butyl-Toyopearl</td>
<td>28.4</td>
<td>14,720</td>
<td>518</td>
<td>129</td>
<td>80</td>
</tr>
<tr>
<td>DEAE-Toyopearl</td>
<td>2.63</td>
<td>1,547</td>
<td>588</td>
<td>14</td>
<td>68</td>
</tr>
<tr>
<td>Phenyl-Superose</td>
<td>0.22</td>
<td>200</td>
<td>952</td>
<td>1.8</td>
<td>118</td>
</tr>
<tr>
<td>Hydroxyapatite</td>
<td>0.02</td>
<td>8</td>
<td>3,242</td>
<td>0.6</td>
<td>377</td>
</tr>
</tbody>
</table>

Results of the purification procedure are shown in Table I. The hydroxylase purified 377-fold in a 0.6% yield showed one major band (Mr 37,700±200; mean of 17 measurements±S.D.) after SDS-15% polyacrylamide gel electrophoresis and silver staining (Fig. 1A). Because the native Mr of the hydroxylase is 41,000±1,000,² this enzyme must act catalytically as a monomer. Other 2-oxoglutarate-dependent dioxygenases (e.g. the deacetoxycephalosporin C synthetase DAOCS) of *Streptomyces clavuligerus*²) and the DAOCS/deacetylcephalosporin C synthetase of *Cephalosporium acremonium*²) also are monomeric enzymes. The pI of the hydroxylase, as determined by nondenaturing isoelectric focusing (Fig. 1B), is 4.6.
Subsequently, approximately 100 µg of homogeneous hyoscyamine 6β-hydroxylase was prepared after several rounds of purification. When the purities of the final enzyme preparations were not adequate, the preparations were further purified by reversed-phase high performance liquid chromatography (HPLC) on...
a Cosmosil 3C8-300 column (4.6×150 mm; Nacalai Tesque, Kyoto). Protein was eluted from the column at 35°C at a flow rate of 1 ml/min with a linear gradient of acetonitrile (30 to 70% in 40 min) containing 0.1% (v/v) trifluoroacetic acid.

Part of the homogeneous enzyme was hydrolyzed in 6N HCl containing 4% (v/v) thioglycolic acid. Its amino acids were analyzed by the ninhydrin method on a Hitachi Amino Acid Analysis System. The amino acid composition is given in Table II. The composition shows no notable characteristics, but the number of arginine residues is low in comparison to the average occurrence of amino acid residues in proteins.9) Direct amino terminal sequencing of the protein was not possible because of negligible yields on an amino acid sequencer (Applied Biosystems), presumably because of amino terminal blockage. Therefore, after digestion of the protein with lysylendopeptidase, its internal peptides were separated by HPLC then analyzed by the sequencer. On the basis of the amino acid sequences of several of the peptides, oligonucleotide probes were synthesized and cDNA clones that encoded hyoscymine 6β-hydroxylase were isolated from a cDNA library made from the mRNA of cultured H. niger roots (unpublished results). We now are making monoclonal antibodies against this hydroxylase using the purified enzyme as the antigen. The availability of homogeneous hyoscymine 6β-hydroxylase provides new opportunities for the study of scopolamine biosynthesis at the molecular level.

References