Note

Bacterial Production and Purification of Phosphorylatable Phosphoenolpyruvate Carboxylase from Tobacco

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Tobacco phosphoenolpyruvate carboxylase (PEPC) [EC 4.1.1.31] cDNA was efficiently expressed in E. coli under the control of the lacZ promoter. The enzyme, purified to homogeneity, had the same catalytic activity, and was phosphorylated in vitro by maize PEPC kinase.

Key words: bacterial expression; cDNA; phosphoenolpyruvate carboxylase; phosphorylation; tobacco

Phosphoenolpyruvate carboxylase (PEPC) [EC 4.1.1.31] catalyzes the β-carboxylation of phosphoenolpyruvate (PEP) to yield oxaloacetic acid and inorganic phosphate. In higher plants, there are multiple isoforms of PEPC1,2; C₂ and CAM isoforms catalyzing the initial carbon dioxide fixation of photosynthesis, and non-photosynthetic C₃ isoforms. It has been shown that kinetic properties of PEPCs differ among C₂ and C₃ plants, especially in respect to the affinity for PEP.3,4) The θ₀ of a purified enzyme from maize (C₄) was 1.16 mm5) which is much higher than that from tobacco (C₃) being 0.06 mm.6) The activity of PEPC is affected by allosteric effectors such as malate and glucose-6-phosphate. The sensitivities of PEPC to the allosteric effectors also differ between C₃ and C₄ plants.7) In addition, the maximal velocity and sensitivities to allosteric effectors change upon post-translational phosphorylation.1,8,9) C₂ PEPC is phosphorylated light-dependently, and the resulting phosphorylated enzyme acquires higher specific activity and lower sensitivity to malate.8) Non-photosynthetic or C₄ PEPC has also been reported to be phosphorylated,10) but its effects are not clear yet. In spite of the complicated regulation of PEPC, there has so far been little information on the three-dimensional structure of PEPC, which may be of great help to clarify the control mechanism of this enzyme. Here, we report bacterial production and purification of recombinant tobacco PEPC which can be analyzed by X-ray crystallography in the near future.

An expression plasmid, named pTE6 was constructed, by inserting the entire coding sequence of tobacco PEPC cDNA11) under the lacZ promoter of pTV118N (Takara Shuzo Co.). In pTE6, the first methionine of lac Zα was replaced with that of tobacco PEPC to synthesize authentic tobacco PEPC protein. The construction procedure is summarized in Fig. 1. E. coli, strain JMI09 was transformed with pTE6, and cultured in LB medium. When cells were grown at 37°C, the PEPC activity in cell-free extract was not increased because most of the recombinant tobacco PEPC was insoluble, forming inclusion bodies (data not shown). However, when bacteria were cultured at 25°C, the enzyme activity derived from recombinant PEPC was observed in the assay mixture without dioxane, an artificial activator of E. coli PEPC (Table I). Bacterial production of tobacco PEPC was confirmed by immunoblot analysis. On a SDS-gel stained by CBB, extracts of bacteria harboring pTE6 showed a distinct polypeptide, of which the molecular weight is approximately 110 kDa which reacted with antiserum to tobacco PEPC (Fig. 2). The same mobility of recombinant PEPC with that of tobacco leaves in SDS-PAGE indicates the recombinant PEPC being correctly translated.

Table 1. Specific Activity of PEPC in Cell Free Extract of E. coli Cultured at 25°C

<table>
<thead>
<tr>
<th>Plasmid</th>
<th>pTV118N</th>
<th>pTE6</th>
</tr>
</thead>
<tbody>
<tr>
<td>- Dioxane*</td>
<td>&lt;0.01</td>
<td>0.27</td>
</tr>
<tr>
<td>+ Dioxane</td>
<td>0.35</td>
<td>0.53</td>
</tr>
</tbody>
</table>

* Dioxane is known as an artificial activator of E. coli PEPC. Without dioxane, bacterial PEPC does not show activity.

Fig. 1. Construction of the Expression Plasmid, pTE6.

The DNA sequence including the first codon of tobacco PEPC (AAATGG) in pTN1003 was altered to an Nco I site (CCATGG) by PCR. The amplified DNA was inserted into a plasmid vector pTV118N at generated Nco I and an internal Sal I sites. This plasmid was designated as pNS7. The remaining portion of tobacco PEPC cDNA in pTS1 was ligated to pNS7 to yield a final plasmid pTE6, that codes for entire sequence of tobacco PEPC cDNA.
Fig. 2. Detection of Recombinant Tobacco PEPC Polypeptide. Cell extracts of E. coli harboring pT181N (lane 2) and pTE6 (lane 3) were run on SDS-PAGE followed by CBB staining (A) and immuno-blots (B). Lane 1 in panel A is the molecular weight marker. Crude extract of tobacco leaves was put on lane 1 in panel B to compare the molecular weight and cross reactivity. Amounts of PEPC activity put on lanes 1 and 3 were the same.

Table II. Purification Summary of PEPC Produced in E. coli

<table>
<thead>
<tr>
<th>Purification step</th>
<th>Activity (units)</th>
<th>Protein (mg)</th>
<th>Sp. activity (units/mg)</th>
<th>Yield (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Crude extract</td>
<td>25.5</td>
<td>11.6</td>
<td>2.2</td>
<td>100</td>
</tr>
<tr>
<td>Ammonium sulfate</td>
<td>20.1</td>
<td>5.2</td>
<td>3.9</td>
<td>79</td>
</tr>
<tr>
<td>Hydroxylapatite</td>
<td>9.2</td>
<td>0.65</td>
<td>14.1</td>
<td>36</td>
</tr>
<tr>
<td>Mono Q</td>
<td>7.4</td>
<td>0.26</td>
<td>28.5</td>
<td>29</td>
</tr>
</tbody>
</table>

Recombinant PEPC was purified from 100 ml of E. coli culture.

For purification of tobacco PEPC, E. coli, PCR1,12] a PEPC deficient mutant strain, was used as the host. When the OD_{600} of the culture reached 0.2, IPTG (40 μM) was added, and the bacteria were grown for 16 h more at 25°C. Bacterial cells were harvested by centrifugation, suspended in extraction buffer (100 mM Tris–HCl pH 7.5, 10 mM MgCl₂, and 10 mM β-mercaptoethanol) and disrupted by sonication. Crude extract was obtained by subsequent centrifugation to remove cell debris, and this was fractionated with ammonium sulfate (0–60% saturation). The precipitate was dissolved in phosphate buffer (50 mM K-phosphate pH 7.5 and 10 mM β-mercaptoethanol) and desalted with Sephadex G-25. The enzyme solution was put on the hydroxylapatite (Bio gel HTP, Bio-rad) column equilibrated with the phosphate buffer. After washing with the phosphate buffer, the enzyme was eluted with a linear gradient of phosphate (50–500 mM). Fractions containing enzymatic activity were concentrated by ammonium sulfate precipitation and desalted with Sephadex G-25. The PEPC protein was put on a Mono-Q (Pharmacia) column and eluted with a linear gradient of NaCl (0–500 mM). Purification data is summarized in Table II. The purified protein was detected as a single fraction on SDS-PAGE (Fig. 3A).

Fig. 3. In Vitro Phosphorylation of Recombinant Tobacco PEPC. Purified recombinant tobacco PEPC (3 μg) and partially purified maize PEPC (5 μg) were incubated in the presence of 0.5 mM ATP (containing trace of [32P]ATP) and 15% glycerol at 30°C for 1 hour. The reaction mixture was separated by 7.5% SDS-PAGE followed by CBB staining (A) and autoradiography (B). Lane 1, molecular weight markers; lane 2, reaction products without PEPC; lane 3, reaction products with complete components; lane 4, reaction products without PEPC.

The N-terminal sequence of the purified enzyme was identical to the deduced amino acid sequence of the tobacco PEPC cDNA (2 to 13). The specific activity of purified recombinant PEPC was 28.5 units/mg, being equivalent to that of the plant source, 32 units/mg,6 indicating that the recombinant PEPC was the same catalytic efficiency as the plant enzyme.

PEPC kinase (PEPCK) was partially purified from maize green leaves using Blue-Sepharose (Pharmacia) according to the procedure of Jiao and Chollet.13) Purified recombinant tobacco PEPC was reconstituted and assayed using PEPC and 32P-ATP. Phosphorylation of tobacco PEPC was analyzed with SDS-PAGE and autoradiography. As shown in Fig. 3, recombinant tobacco PEPC was phosphorylated in vitro by maize PEPCK. The fact is consistent with our previous findings that a specific serine residue, which corresponds to phosphorylated serine of maize C₆ PEPC, is in the sequence of tobacco PEPC,11 and with a report that purified tobacco PEPC was phosphorylated by PEPCKs from leaves of tobacco and maize.14)

Using this recombinant tobacco PEPC, the tertiary structure will soon be identified by X-ray crystallography.

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References
Bacterial Production of Tobacco PEP Carboxylase