Note

Efficient Method for the Preparation of Escherichia coli Polynucleotide Phosphorylase Suitable for the Synthesis of Polynucleotides

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Polynucleotide phosphorylase (PNPase, polynucleotide: ortho-phosphate nucleotidyltransferase, EC 2.7.7.8) catalyzes a reversible reaction in the following way:

\[ n \text{ ribonucleoside-P-P} \rightleftharpoons (\text{ribonucleoside-P})_n \text{+} n \text{ Pi} \]

The enzyme has been used for the synthesis of biologically active polynucleotides such as poly(I)-poly(C), an inducer of interferon formation. Especially the enzyme from *Escherichia coli* was reported to be able to synthesize various kinds of homo- and copolymers of nucleotides. However, bacterial cells are usually poor in PNPase and rich in both nucleases and nucleoside diphosphate-degrading enzymes, and the enzyme must be purified with tedious procedures. Therefore, we have established an efficient production system by the amplification of the enzyme with recombinant DNA techniques in *E. coli*.

From *E. coli*, the pnp gene coding for PNPase was first cloned and characterized by Régnier et al. On the basis of their sequence data, we synthesized primer DNA A and B for PCR-cloning of the pnp gene (see Fig. 1). The sequences of primer DNAs were designed directly to construct the *tac* promoter gene by using plasmid pDR540. The coding region of the pnp gene amplified by PCR was separated and purified by agarose gel electrophoresis. After digesting the purified product with BamHI, the resultant DNA fragment was inserted into the BamHI site of pDR540 in order to overexpress the pnp gene under the control of the tac promoter. The constructed plasmid, pDR-PNP, was introduced into *E. coli* JM105 cells.

JM105 cells harboring pDR-PNP was cultivated at 37°C to 5 x 10^8 cells/ml in 2 x YT medium supplemented with 100 μg/ml of ampicillin. Then IPTG (final 1 mM) was added to the culture for the induction of cloned pnp gene. The pnp gene product, PNPase, was analyzed by SDS-polyacrylamide gel electrophoresis (9% polyacrylamide gel) of the whole cellular protein. The amount of PNPase (MW 86,000) increased and reached about 10% of total cellular protein after 18 h of induction (Fig. 2A, lane 2). On the contrary, no significant level of PNPase was detected even in the cells without induction (lane 1) or in cells carrying pDR540 (lane 3). As shown in Table 1, the IDP polymerization activity of crude extract from cells carrying pDR-PNP was about 150-fold higher than that from cells carrying pDR540.

We next devised a simple method for preparation of the enzyme with a high yield from the recombinant cells by the following operations. All the operations were done at 4°C, unless otherwise indicated. Centrifugation was at 15,000 x g for 10 min.

**Step 1. Extraction.** From 500 ml of cell culture grown in 2 x YT medium for 18 h with IPTG, cells were collected by centrifugation and suspended in 100 ml of a buffer containing 50 mM Tris-HCl (pH 7.8), 5 mM MgCl₂, and 10 mM 2-mercaptoethanol. A crude extract was prepared with

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**Fig. 1.** Construction of pDR-PNP.

Chromosomal DNA of *E. coli* C600 (r-, m-, supE) was purified by the method of Saito and Miura. Primer A and B were synthesized for PCR-cloning of the coding region of pnp gene by using Gene Assembler Plus DNA synthesizer (Pharmacia Co.). PCR was done as described in the user's manual of a GeneAmp PCR Reagent Kit with AmpliTaq DNA polymerase (Perkin Elmer Cetus). PCR products were purified by electrodialysis from an agarose gel and cleaved with BamHI. The BamHI-DNA fragment was introduced into the BamHI site downstream from the tac promoter of pDR540. The resultant plasmid was named pDR-PNP. Plasmids are shown as linear forms opened at the HindIII site. Open boxes, closed boxes, “bla”, and “ori” indicated the pnp coding region, the tac promoter, β-lactamase gene, and replication origin of the plasmid, respectively.

Abbreviations: IPTG, isopropyl-β-D-thiogalactopyranoside; kb, kilo base pair(s); PCR, polymerase chain reaction; PNPase, polynucleotide phosphorylase.

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**Fig. 2.** SDS-Polyacrylamide Gel Electrophoresis.

SDS-9% polyacrylamide gel electrophoresis was done as described by Laemmli. The arrow indicates the position of PNPase; and the molecular size markers are shown in the left-side. (A) Whole cellular protein of recombinant cells cultivated for 18 h with or without IPTG. Lanes 1, JM105 cells carrying pDR-PNP without IPTG; lane 2, JM105 cells carrying pDR-PNP with IPTG; lane 3, JM105 cells carrying pDR540 with IPTG. (B) Enzyme preparations form JM105 cells carrying pDR-PNP. Lane 1, whole cellular protein; lane 2, crude extract; lane 3, ammonium sulfate fraction; lane 4, DEAE-Sephadex A-25 fraction; lane 5, Sephadex G-200 fraction.
Table I. Polynucleotide Phosphorylase Activity of Crude Extract from Recombinant Cells

Recombinant cells were cultivated with 1 mM IPTG for 18 h and harvested with centrifugation. Cells were disrupted by sonic oscillation and spun down. PNPase activity of the resultant crude extracts was measured as described by Rokugawa et al. 12

<table>
<thead>
<tr>
<th>Strain</th>
<th>Polynucleotide phosphorylase activity (units/mg protein)</th>
</tr>
</thead>
<tbody>
<tr>
<td>JM105 [pDR540]</td>
<td>0.02</td>
</tr>
<tr>
<td>JM105 [pDR-PNP]</td>
<td>3.17</td>
</tr>
</tbody>
</table>

* 1 unit = 1 μmol of IDP incorporation per min.

Table II. Purification of Polynucleotide Phosphorylase

<table>
<thead>
<tr>
<th>Step</th>
<th>Total protein (mg)</th>
<th>Total activity (units)</th>
<th>Specific activity (units/mg protein)</th>
<th>Yield (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Crude extract</td>
<td>475.0</td>
<td>1450.0</td>
<td>3.05</td>
<td>100.0</td>
</tr>
<tr>
<td>Ammonium sulfate</td>
<td>329.8</td>
<td>1258.0</td>
<td>3.79</td>
<td>86.5</td>
</tr>
<tr>
<td>DEAE Sephadex A-50</td>
<td>45.0</td>
<td>893.0</td>
<td>19.83</td>
<td>61.5</td>
</tr>
<tr>
<td>Sephadex G-200</td>
<td>23.0</td>
<td>634.0</td>
<td>27.50</td>
<td>43.7</td>
</tr>
</tbody>
</table>

Centrifugation followed by sonic oscillation.

Step 2. Ammonium sulfate precipitation. The crude extract was fractionated with ammonium sulfate precipitation. Materials precipitated at 0.2 to 0.6 saturation were collected by centrifugation. Precipitates were dissolved in 15 ml of a buffer containing 50 mM Tris-HCl (pH 7.8), 5 mM MgCl₂, 10 mM 2-mercaptoethanol, and 5% glycerol (hereafter referred to as the standard Tris buffer), and dialyzed against the same buffer.

Step 3. DEAE-Sephadex A-50 chromatography. The dialyzed solution was put on a DEAE Sephadex A-50 column (25 mm diameter × 250 mm) equilibrated beforehand with a 200 ml of the standard Tris buffer. After the column was washed with the standard buffer, elution was done with a linear gradient of NaCl in the standard Tris buffer (0.1 M to 0.4 M, total volume, 400 ml). The enzyme activity was monitored by IDP polymerization. The enzyme was concentrated by precipitation with ammonium sulfate as described in step 2. The precipitates were dissolved in the standard Tris buffer (3.0 ml).

Step 4. Sephadex G-200 chromatography. The sample was put directly on a Sephadex G-200 column (25 mm diameter × 840 mm) equilibrated with the standard Tris buffer. The column was eluted with the same buffer and active fractions were combined. The combined enzyme preparation (20 ml) showed about 9-fold purification over the starting crude extract with over a 40% yield (see Table II). The result enzyme preparation was homogeneous on SDS-polyacrylamide gel electrophoresis (Fig. 2B, lane 5). It also showed neither nuclease activity nor nucleoside di-phosphate-degrading enzyme activity.

By using the enzyme preparation, we tried to synthesize several homopolymers, and confirmed that the polymers with a high molecular weight could be efficiently formed. The PNPase-producing system described here would allow us readily to obtain a large amount of the enzyme preparation suitable for practical production of polynucleotides.

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References