Polynucleotide Phosphorylase from Acromobacter sp. KR 170-4

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Eighty-five strains of bacteria were screened for selection of microorganisms suitable for industrial production of polynucleotides. Among these bacteria, Acromobacter sp. KR 170-4 (ATCC 21942) was found to be rich in polynucleotide phosphorylase (PNPase) in its "salt-shockate" as compared with the other strains tested. PNPase was purified about 50-fold from the "salt-shockate" of Acromobacter sp. KR 170-4, and properties of the enzyme were elucidated. Optimal pH for reaction was 10.1. Stable pH range at 37°C was between pH 6.5 and 10.5. Optimal temperatures were 46°C for polymerization of ADP or IDP, and 43°C for CDP or UDP. The enzyme was stable below 55°C at pH 9.2. The enzyme required Mn²⁺ rather than Mg²⁺ unlike the other PNPases reported. Optimal concentration of Mn²⁺ was 6 mm.

In the previous paper, it was demonstrated that polynucleotides could be synthesized from nucleoside diphosphates by cultures or cell-extracts of microorganisms belonging to genera Pseudomonas, Serratia, Xanthomonas, Proteus, Aerobacter, Bacillus, and Brevibacterium. These strains were rich in polynucleotide phosphorylase (PNPase) easily extractable from cells, and poor in both nuclease and nucleoside-diphosphate-degrading enzymes. Subsequently, Acromobacter sp. KR 170-4 (ATCC 21942) was found to be superior to the above-mentioned bacteria in productivity of PNPase extractable from the cells. The strain was also poor in both nuclease and nucleoside-diphosphate-degrading enzymes. The present paper deals with the purification and properties of PNPase from the strain of Acromobacter.

MATERIALS AND METHODS

Microorganisms. Acromobacter sp. KR 170-4 (ATCC 21942) was used in this study. This strain had been isolated by K. Rokugawa, one of the authors, from "Kamaboko" (a kind of fish-paste) prepared in Sendai in 1967.

Materials. ADP, IDP, CDP and UDP were prepared in this laboratory. DEAE Sephadex A-50 and Sepharose 6B were obtained from Pharmacia Fine Chemicals Co. Carrier ampholyte for isoelectric focusing was a product of LKB, Stockholm-Bromma, Sweden.

Medium. Yamasa Extract medium, containing 50 ml of "Yamasa Extract L" and 8 g of yeast extract in 1 liter of water, pH 7.0, was used. "Yamasa Extract L" is a koji-mold extract supplemented with 20% of NaCl, 5% of monosodium glutamate, 5% of yeast extract, 0.5% of succinate, 0.25% of 5'-IMP and 0.25% of 5'-GMP.

Cultivation in a jar fermenter. Bacetrial cells were grown in 100 ml of the medium with shaking at 28°C for 18 hr in a Sakaguchi flask. The seed culture (300 ml) was transferred to a jar fermenter containing 15 liters of the medium, and incubated with forced aeration at 28°C for 6 hr. The pH was kept at between 6.8 and 7.2 during the incubation. From the culture, 154 g of the wet cells were harvested.

Extraction of the enzyme. Extraction of the enzyme from the cells were done by the salt-shock method described in the previous paper. DeAE Sephadex A-50 and Sepharose 6B were obtained from Pharmacia Fine Chemicals Co. Carrier ampholyte for isoelectric focusing was a product of LKB, Stockholm-Bromma, Sweden.

Assay of PNPase. The estimation of PNPase activity was based on the polymerization reaction. The reaction mixture contained 48 μmoles of IDP (Na), 5 μmoles of MnCl₂, 1.0 ml of Tris-HCl buffer (pH 9.2), 0.5 ml of enzyme solution and 0.5 ml of water. The mixture was incubated at 37°C for 60 min. To 0.5 ml of the reaction mixture, 0.5 ml of uranyl reagent (10% of perchloric acid containing 0.3% uranyl acetate) was added. After allowing to stand for 15 min in ice,
the mixture was centrifuged. The supernatant was
diluted 250-fold with 0.01 N HCl, and absorbance at
250 nm was read. A unit of PNPase was defined as
the amount of enzyme, which, under the above condi-
tion, polymerized 1 μmole of IDP per 1 min.

Isoelectric focusing in pH gradient. Isoelectric
focusing was performed according to Vesterberg et al.21
with carrier ampholyte (pH 3–10) in a LKB column.

Protein determination. Protein was determined by
the method of Lowry et al.3

RESULTS

Selection of microorganisms suitable for pro-
duction of polynucleotides

Eighty-five strains of bacteria were screened
for selection of microorganisms suitable for
industrial production of polynucleotides. Among
these bacteria, Achromobacter sp. KR 170–4 (ATCC 21942) was found to be the
highest in PNPase in the “salt-shockate” as
compared with the other strains tested. Ap-
parent activity of PNPase in “salt-shockate” of
several strains is shown in Table I. The ap-
parent activity of E. coli B, one of the well-
known PNPase sources, was fairly low, pro-
bably because the enzyme preparation would be
rich in nucleoside-diphosphate-degrading en-
zymes and nucleases, both of which interfere
with polynucleotide synthesis.

Purification of the enzyme

As the result of screening, PNPase activity
in the “salt-shockate” of Achromobacter sp.
KR 170–4 (ATCC 21942) was the highest in
the strains tested. Then, purification of
PNPase from the culture of this strain was
attempted. All operations were carried out at 3°C, unless otherwise indicated.

Step 1. “Salt-shockate” (500 ml) was
fractionated with ammonium sulfate precipi-
tation at 0°C. Materials precipitated at
0~0.3 saturation (fraction A), 0.3~0.4 saturation
(fraction B), 0.4~0.6 saturation (frac-
tion C), 0.6~0.8 saturation (fraction D)
and 0.8~1.0 saturation (fraction E) were col-
lected successively by centrifugation. Each of
precipitates was dissolved in 0.1 M Tris-HCl
buffer, pH 8.0, containing 10–3 M mercapto-
ethanol and 10–3 M Mn2+ (hereafter referred to
as the Tris buffer), and dialyzed against the
same buffer. The PNPase activity was re-
covered in two fractions; fraction B and frac-
tion C. As the specific activity of fraction B
was higher than fraction C, the former was
used in the following purification process.

Step 2. The above fraction B (40 ml)
was placed on a DEAE Sephadex A–50 column
(2.5×45 cm) equilibrated beforehand with
the Tris buffer. After washing the column
with 200 ml of the same buffer, elution was
carried out stepwise with 500 ml of 0.4 M NaCl
and 150 ml of 1.0 M NaCl in the same buffer
at a flow rate of 30 ml per hr, and 5 ml-fra-
tions were collected. Fractions having activity
(No. 90 to No. 100) were pooled, concentrated
with polyethylene glycol # 6,000, and dialyzed
against the same buffer.

Step 3. Material from step 2 (25 ml) was
placed on a second DEAE Sephadex A–50
column (1.93×28 cm) equilibrated with the
Tris buffer. After washing the column with

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**Table I. Apparent Activity of Polynucleotide Phosphorylase in “Salt-shockate” of Several Microorganisms**

<table>
<thead>
<tr>
<th>Strain</th>
<th>Activity (U/mg protein) ×10^2</th>
</tr>
</thead>
<tbody>
<tr>
<td>Achromobacter sp. KR 170–4</td>
<td>34.0</td>
</tr>
<tr>
<td>(ATCC 21942)</td>
<td></td>
</tr>
<tr>
<td>Pseudomonas sp. R–399</td>
<td>26.5</td>
</tr>
<tr>
<td>(ATCC 21638)</td>
<td></td>
</tr>
<tr>
<td>Pseudomonas putrefaciens</td>
<td>25.2</td>
</tr>
<tr>
<td>(IFO 3908)</td>
<td></td>
</tr>
<tr>
<td>Xanthomonas begoniae</td>
<td>20.2</td>
</tr>
<tr>
<td>(IAM 1644)</td>
<td></td>
</tr>
<tr>
<td>Micrococcus lysodeikticus</td>
<td>16.7</td>
</tr>
<tr>
<td>(IAM 1313)</td>
<td></td>
</tr>
<tr>
<td>Achromobacter delmarvae</td>
<td>15.2</td>
</tr>
<tr>
<td>(IFO 12668)</td>
<td></td>
</tr>
<tr>
<td>Escherichia coli B</td>
<td>8.7</td>
</tr>
<tr>
<td>(IAM 1062)</td>
<td></td>
</tr>
</tbody>
</table>
100 ml of the same buffer, the enzyme was eluted with a linear NaCl gradient from 0.2 to 0.5 M prepared in 800 ml of the same buffer. Fractions having activity (No. 17 to No. 23) were pooled, concentrated, and dialyzed against the same buffer.

**Step 4.** Post-DEAE Sephadex A-50 enzyme (12 ml) was applied to a Sepharose 6B column (2.5 x 48.5 cm) equilibrated with the Tris buffer. Enzyme was eluted with 250 ml of the same buffer and fractions containing activity (30 ml) were pooled, concentrated and dialyzed as mentioned above.

**Step 5.** The enzyme from Step 4 (3 ml) was applied to the same column as employed in step 4 (Fig. 1). The purified enzyme was concentrated, dialyzed against 0.02 M Tris- HCl buffer (pH 8.0) containing 10^{-3} M mercaptoethanol and stored at -20°C.

Data from a typical enzyme purification are shown in Table II. The final preparation represented about 50-fold purification over the starting “salt-shockate” and contained about 7.3% of the activity present. The purified enzyme preparation showed high PNPase activity, but neither nuclease nor nucleoside diphosphate-degrading enzyme activities.

**Isoelectric focusing of the partially purified enzyme preparation**

The partially purified enzyme preparation (corresponding to the post DEAE Sephadex enzyme) was subjected to isoelectric focusing. As shown in Fig. 2, the isoelectric point of

![Fig. 1. Second Gel Filtration on Sepharose 6B.](image)

The enzyme solution eluted from 1st Sepharose 6B column was concentrated to 3 ml and loaded on a 2.5 x 48.5 cm column of Sepharose 6B which was previously equilibrated with the Tris buffer. The enzyme was eluted with the same buffer at a flow rate of 10 ml per hr and 5 ml-fractions were collected. ○——○, absorption at 280 nm; ●—●, PNPase activity.

PNPase was observed at pH 4.8. During the focusing, however, appreciable precipitate was formed and simultaneously significant inactivation of the enzyme was observed. Therefore, this technique could not be used for the enzyme purification.

**Effect of metal ions on enzyme activity**

Divalent metal ions were essential for the
TABLE II. PURIFICATION OF POLYNUCLEOTIDE PHOSPHORYLASE
FROM Achromobacter sp. KR 170-4

<table>
<thead>
<tr>
<th>Step</th>
<th>Protein (mg)</th>
<th>Total activity (U)</th>
<th>Specific activity (U/mg protein)</th>
<th>Yield (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Salt- shockate</td>
<td>14,564.2</td>
<td>1,019.5</td>
<td>0.07</td>
<td>100</td>
</tr>
<tr>
<td>1. (NH₄)₂SO₄ Fractionation</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>fraction B</td>
<td>1,040.6</td>
<td>385.3</td>
<td>0.37</td>
<td>37.8</td>
</tr>
<tr>
<td>fraction C</td>
<td>1,857.6</td>
<td>516.1</td>
<td>0.28</td>
<td>50.6</td>
</tr>
<tr>
<td>2. 1st DEAE-Sephadex</td>
<td>507.4</td>
<td>297.3</td>
<td>0.59</td>
<td>29.1</td>
</tr>
<tr>
<td>3. 2nd DEAE-Sephadex</td>
<td>245.6</td>
<td>176.6</td>
<td>0.72</td>
<td>17.3</td>
</tr>
<tr>
<td>4. Sepharose 6B</td>
<td>46.8</td>
<td>99.1</td>
<td>2.11</td>
<td>9.7</td>
</tr>
<tr>
<td>5. Gel-filtration repeated</td>
<td>21.6</td>
<td>74.3</td>
<td>3.44</td>
<td>7.3</td>
</tr>
</tbody>
</table>

PNPase activity. Mn²⁺ was found to activate the enzyme more effectively than Mg²⁺ (Fig. 3). The optimal concentration of Mn²⁺ was found to be 6 mM. On the other hand, even in the presence of more than 10 mM of Mg²⁺ the reaction rate was less than 60% of that in the optimal concentration of Mn²⁺

**Fig. 3. Effect of Divalent Cation Concentration on the Rate of Polymerization Reaction.**

Each tube contained 2.5 x 10⁻² units of enzyme, 24 μmoles of IDP (Na), 2.5 μmoles of MnCl₂ (closed circle) or MgCl₂ (open circle) at pH 9.2 in a final volume of 1.0 ml. The reaction mixture was incubated at 37°C for 60 min.

**Effect of pH on enzyme activity**

The optimal pH for the polymerization reaction was found to be 10.1 (Fig. 4). Stable pH ranges were estimated from the residual activity after incubation of the enzyme at various pH for 1 hr at 37° and 50°C (Fig. 5). The enzyme was more stable in alkaline condition than in neutral or acid condition: The enzyme solution became turbid below pH 6.5 and precipitated below pH 5.5, where the enzymatic activity was completely lost. The stable pH range at 37°C was between pH 6.5 and 10.5, and even at 50°C, the enzyme was stable between pH 7.0 and 9.0.

**Effect of temperature on enzyme activity**

The effect of temperature on the rate of reaction was estimated for different substrates; ADP, IDP, CDP and UDP (Fig. 6). Slight variations in the optimal temperatures were...
PNPase from *Achromobacter* sp. KR 170–4

FIG. 5. The Stability of PNPase to pH.

70 μg of enzyme (2.4 × 10⁻¹ units) was preincubated at different pH in 0.1 ml of M/35 veronal buffer for 1 hr at 37°C (open circle) or 50°C (closed circle). Each of enzyme solutions was then cooled to 0°C and the residual activity was determined.

Fig. 5 shows the stability of PNPase to pH. The enzyme was incubated at different pH values (5 to 12) for 1 hour at 37°C (open circle) or 50°C (closed circle). After cooling to 0°C, the residual activity was determined.

FIG. 6. Effect of Temperature on the Rate of Polymerization Reaction.

The reaction mixture contained 2.5 × 10⁻² units of enzyme, 100 μmoles of Tris-HCl buffer (pH 9.2), 2.5 μmoles of MnCl₂, and 24 μmoles of ADP, IDP, CDP or UDP in a final volume of 1.0 ml. The incubation time was 1 hr at indicated temperatures. ○−○, ADP; ●−●, IDP; ■−■, CDP; △−△, UDP.

**DISCUSSION**

PNPase has been reported to be widely distributed in microorganisms⁴,⁵ and even in animal tissues.⁶ In particular, *Azotobacter vinelandii*, *Clostridium perfringens*, *Escherichia coli* and *Micrococcus lysodeikticus* have been well known as representative PNPase-producing bacteria, and the properties of PNPase from these bacteria have been studied in detail.⁴,⁷−¹² In the previous paper,¹³ it was demonstrated that several bacteria such as *Pseudomonas* sp. R–399 were superior to *E. coli* in PNPase activity. In this paper, *Achromobacter* sp. KR 170–4 was proved to have much higher PNPase activity than the above-mentioned bacteria in the "salt-shockate." Probably *Achromobacter* sp. KR 170–4 is one of the best strains for preparing polynucleotides.

Several properties of PNPase purified about
50-fold from the "salt-shockate" of *Achromobacter* sp. KR 170-4 will be compared with those of well-known microbial PNPases:

It has been generally believed that Mg$^{2+}$ is required for the reaction catalyzed by PNPase. Babinet *et al.*\(^{10}\) have demonstrated that Mg$^{2+}$ can be replaced by Mn$^{2+}$, Co$^{2+}$, Zn$^{2+}$ and Cd$^{2+}$ in the polymerization reaction catalyzed by PNPase from *A. vinelandii*, *E. coli* and *M. lysodeikticus*, although the efficiencies of the various ions are quite different. Katoh *et al.*\(^{1}\) have also demonstrated that Mg$^{2+}$ can be replaced by Mn$^{2+}$ and Co$^{2+}$ without significant loss of efficiency in the polymerization reaction with *Pseudomonas* sp. R-399 system. On the other hand, polymerization of GDP is reported to occur in the presence of Mn$^{2+}$, if the temperature of the incubation is raised to 60°C in *E. coli* system.\(^{13}\) It is of particular interest that *Achromobacter* PNPase rather specifically requires Mn$^{2+}$ under normal conditions and Mg$^{2+}$ can not completely replace Mn$^{2+}$. In addition, Hsieh *et al.*\(^{14}\) have demonstrated that Mn$^{2+}$ was essentially required for the polymerization reaction by a particular PNPase from *E. coli* Q13, a mutant which has been reported to be defective in RNase I and PNPase, and Mg$^{2+}$ can not replace Mn$^{2+}$

The optimal pH for the polymerization reaction of *Achromobacter* PNPase was 10.1. This value seems to be a little higher than those of well-known PNPase. For example, pH optima of PNPase from *A. agilis* and *M. lysodeikticus* are reported to be 7.5 to 9 and 9 to 10, respectively.\(^{4,7}\) This fact may be related to the marked stability of the *Achromobacter* enzyme in alkaline condition. The enzyme is stable below 55°C at pH 9.2, whereas Lucas *et al.*\(^{9}\) have demonstrated that *E. coli* PNPase is stable below 55°C at pH 8.2.

The optimal temperature for the PNPase activity varies with the substrates: generally purine nucleotides require higher temperature than pyrimidine nucleotides. In *E. coli* system, the optimal temperature for the polymerization of ADP and GDP is about 60°C, while that for CDP and UDP is between 45 and 55°C.\(^{9}\) Similar tendency is also observed in *Achromobacter* PNPase, although the optimal temperature thereof is lower than that of *E. coli* enzyme and the effect of base on the optimal temperature in *Achromobacter* PNPase is not so much as in *E. coli* enzyme.

It may be concluded that in general enzymatic properties, PNPase from *Achromobacter* sp. KR 170-4 has similarities to well-known enzymes reported, but has difference in the metal ion requirement: the former requires mainly Mn$^{2+}$ while the latter mainly Mg$^{2+}$. The further investigation concerning with the other physical and biochemical properties of the enzyme must be the subject of future research.

REFERENCES