Purification of polynucleotide phosphorylase by affinity chromatography and some properties of the purified enzymes

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ABSTRACT

A method is described for the preparation of p-amino-phenyl oligo(dT)-Sephadex. This matrix has been used for the purification of polynucleotide phosphorylase from both E.coli and B.stearothermophilus. The effects of temperature and pH on the binding of the different enzymes to the matrix have been investigated. B.stearothermophilus isolated by affinity chromatography may be useful in selectively removing the polyA tract on the 3'-end of mRNA's.

INTRODUCTION

The enzyme polynucleotide phosphorylase (E.C.2.7.7.8) has been purified from a wide variety of organisms by conventional techniques. Affinity chromatography has been established as a powerful tool in enzyme purification (1, 2) but until now there have been no reported purifications of polynucleotide phosphorylase by this technique.

The purification of polynucleotide phosphorylase from B. stearothermophilus has been reported recently (3). We wish to report the preparation of p-aminophenyl oligo(dT)-Sephadex and its use in the purification of polynucleotide phosphorylase from Escherichia coli and Bacillus stearothermophilus. By selection of the correct conditions, a 130 fold purification of the enzyme from B.stearothermophilus can be obtained.

MATERIALS AND METHODS

General

E.coli and B.stearothermophilus were obtained as frozen pastes from the Microbiological Research Establishment, Porton. Nucleotide diphosphates and oligoribonucleotides were obtained from P-L Biochemicals Inc. (Up)₅U was obtained from the Boeringer Corp. [¹⁴C]-Nucleotide diphosphates (except IDP) were purchased from the Radiochemical Centre, Amersham.

Preparation of [¹⁴C] IDP

Sodium nitrite (5 mg) was added to a solution of ADP (1 mg/50 µl containing 10µCi of [¹⁴C] ADP) in 10% acetic acid at 0°. The container was stoppered and kept at 0° for 24 hrs before removing the solvent under vacuum. The reaction mixture was applied to a cellulose TLC plate and developed in isobutyric acid/ammonia/water, 66/1/33. The product moved with an R_f = 0.13 and was eluted from the cellulose with 0.5M triethylammonium bicarbonate. The product was chromatographically identical to authentic Inosine diphosphate.

Preparation of p-aminophenyl oligo(dT)-Sephadex

Oligodeoxythymidylate [oligo(dT)] was prepared by chemical polymerisation of thymidine monophosphate (4, 5). Oligo(dT) (Pyridinium salt, 100 mg) was dissolved in dry pyridine (5.0 ml), recrystallised p-nitrophenol (0.60 g) was added and the residue dried by repeated evaporation of pyridine. The residue was redissolved in dry pyridine (10 ml) and dicyclohexylcarbodiimide (0.8 g) added, the mixture was then stirred for 72 hrs at room

temperature in the dark. The pyridine was evaporated under vacuum and the residue dissolved in water. The solution was repeatedly extracted with ethyl acetate (4 x 10 ml) and the aqueous layer evaporated to dryness.

The p-nitrophenyl ester of oligo(dT) was reduced by catalytic hydrogenation using palladium on charcoal (10% w/w, 50 mg) in 50% aqueous methanol (50 ml). The reduction was carried out at room temperature for 1.5 hrs at 35 psi after which the catalyst was removed by filtration and the solution evaporated to dryness. The oligonucleotide was purified on Whatman 3MM paper by elution with 1M ammonium acetate:ethanol. The oligo(dT) remained close to the origin and was eluted from the paper with 0.2M sodium bicarbonate.

The p-aminophenyl oligo(dT) was coupled to Sepharose 4B (350 ml) which had been activated by cyanogen bromide (6). The gel was stored as a suspension in water at 4° in the presence of sodium azide.

Preparation of enzyme extracts

Cells were broken by digestion with lysozyme in the presence of deoxyribonuclease and EDTA (7). After removal of cell debris, nucleic acid and PNPase activity were precipitated with polyethyleneimine (final concentration 0.30%) (8). The pellet was extracted with 0.01M-Tris, 0.001M-EDTA, 2mM-mercaptoethanol, 0.50M-KCl, pH8.2, and the supernatant containing PNPase activity was applied to a column (2.5 x 80 cm) of Sepharose-4B and eluted with the same buffer. Active fractions were pooled and stored at -20°.

Assay of enzyme activity

Polymerisation was measured by following the incorporation of [¹⁴C] ADP into polymeric material (9). The activity of E. coli PNPase was measured at 37° in 0.1M tris, 10mM MgCl₂, 1mM

EDTA, 2mM mercaptoethanol, pH9.2. The B.stearothermophilus PNPase activity was measured at 70° in a 0.1M glycine-sodium hydroxide buffer (pH9.2) containing 10mM MgCl₂, 1mM EDTA, 2mM mercaptoethanol. One unit of activity is defined as the amount of enzyme which will convert 1 μ mole of ADP into polymer.

Phosphorolysis was measured by following the incorporation of ³²Pi into ADP (10). The B.stearothermophilus enzyme was assayed at 70° using a 0.1M glycine buffer (pH8.0) containing 10mM K₂HPO₄, 0.5mM MgCl₂ and 2mM mercaptoethanol. The standard assay mixture also contained 1.5 μ moles of polyA per ml expressed as mononucleotide units, in other studies the polyA was replaced by other polynucleotides at the same concentration.

Before use, all buffers were degassed, purged with nitrogen and degassed again.

Protein was measured by the method of Lowry (11).

Effect of pH and temperature on the binding to the Sepharose derivative

The effect of these parameters was measured using a batch procedure. Aliquots of enzyme were added to a stirred suspension of the gel in a jacketed vessel. After 15 minutes the suspension was rapidly filtered on a sintered glass disc and the filtrate assayed for enzyme activity. In the temperature studies, allowance was made for the change of buffer pH with temperature.

Detection of RNase activity in purified B.stearothermophilus PNPase

Under the polymerisation conditions used, no detectable hydrolysis of [¹⁴C] polyC could be detected (12).

RESULTS

Binding of p-aminophenyl oligo(dT) to CNBr activated Sepharose

When the ligand was reacted with the activated Sepharose, 44% of the material (measured spectrophotometrically) was covalently bound to the matrix. A typical reaction gave a conjugate containing 2.5 μ mole ligand/ml with a capacity of 96 units/ml.

Purification of Polynucleotide Phosphorylase

A typical experiment is shown in Fig. 1. A column (1.0 x 18.0 cm) of p-aminophenyl oligo(dT)-Sepharose was equilibrated at room temperature with 0.1M tris, 10mM $MgCl_2$, 1mM EDTA, 2mM mercaptoethanol, pH8.2. A crude extract (20 ml) of B.stearothermophilus (10 units/mg, 3.6 mg/ml) was applied to the column and washed on with starting buffer at room temperature. Under these conditions, the enzyme binds strongly to the ligand. The column was washed with starting buffer until no more protein was eluted. The buffer then changed to a magnesium free buffer and a linear gradient of potassium chloride applied at a flow rate of 0.5 ml/min. A peak of activity eluted at 1.2M KCl and had a specific activity of 1350 units/mg, representing a 900 fold purification from the cell lysate. The activity can also be eluted with an irrigant containing 1mM ADP. The homogeneity of the protein was checked by polyacrylamide gel electrophoresis. The purification is outlined in Table 1.

Effect of pH and temperature on binding to the matrix

The results of the experiments with the E.coli enzyme are shown in Figures 2 and 3. The binding of the enzyme to the immobilised ligand is strongest at pH8.2 and declines sharply on either side of this value. The effect of temperature is less marked but the binding is strongest at temperatures below

Figure 1. Purification of *B. stearothermophilus* polynucleotide phosphorylase.

A solution of enzyme (20 ml, 3.6 mg/ml) was applied to a column (10 x 18.0 cm) of p-aminophenyl oligo(dT)-Sephacrose which had been equilibrated with 0.1M-Tris, 10mM-MgCl₂, 1mM-EDTA, pH8.2 at room temperature. After elution of unbound protein, enzyme was eluted with a linear gradient of KCl (0-2.0M).

—□—□—□— Protein (mg/ml)
 —▲—▲—▲— Specific Activity (units/mg)

TABLE 1

Steps	Specific Activity units/mg	Purification	Recovery %
1 Cell lysate	1.5	1	100
2 Polyethylene- imine	5.5	3.6	95
3 Sepharose 4B	10	6.7	80
4 Oligo(dT)- Sephacrose	1350 ^a	900	72

^a Peak Fraction

Purification of *B. Stearothermophilus* Polynucleotide Phosphorylase as described in methods and results section. Units are polymerization units.

Figure 2. Effect of pH on the binding of enzyme to ligand.

Aliquots of enzyme were added to suspensions of the ligand-conjugate which had been equilibrated in 0.1M-Tris, 10mM-MgCl₂, 1mM-EDTA at the given pH. Temperature was held constant at 10°.

—□—□— E.coli
 —●—●— B.stearothermophilus

Figure 3. Effect of temperature on the binding of enzyme to the ligand.

Aliquots of enzyme were added to suspensions of the ligand-conjugate which had been equilibrated with 0.1M-Tris, 10mM-MgCl₂, 1mM-EDTA, pH8.2 at the given temperature.

—■—■— E.coli
 —○—○— B.stearothermophilus

10°. Similar results were observed with the enzyme from B. stearothermophilus although the enzyme remained bound to the ligand at higher temperatures.

These results suggested that specific elution of the enzyme can be achieved by selection of the appropriate pH and temperature. This result was confirmed, for example B.stearothermophilus PNPase binds to the ligand at pH8.2 and 5° but when the temperature is raised or the buffer pH changed, the enzyme becomes desorbed.

Properties of the purified B.stearothermophilus enzyme

pH profiles for the phosphorolysis and polymerisation reactions are shown in Figure 4. The pH optima for both reactions agree with those previously reported (3). Substrate specificity is shown in Table 2. The enzyme shows a remarkably strong preference for polyA and ADP in the phosphorolysis and polymerisation reactions. The enzyme is not base specific with respect to primer as (Ip)₅I, (Up)₅U and (Ap)₅A all function as equally good primers for the polymerisation of CDP. Oligo(dT)₁₂ [d(pT)₁₂] has also been used as a primer and functions with one fifth of the efficiency of (Ip)₅I as primer for the polymerisation of CDP (13).

DISCUSSION

Weatherford et al reported that E.coli polynucleotide phosphorylase was retarded on a column of DNA agarose (14). The enzyme was not specifically bound to this material although it was retarded. A conjugate is described here in which the oligonucleotide is covalently bound to the matrix. Oligo(dT) was chosen as a ligand because it is known that polynucleotide phosphorylase binds strongly to oligonucleotides (15) and that the enzyme is competitively inhibited by deoxynucleotides (16). The oligo(dT) used in this work was

Figure 4. pH profiles of phosphorolysis and polymerisation reactions of the enzyme from *B.stearothermophilus*.

Assays were performed as described in the Methods section. Final concentration of ADP in the polymerisation assay was 24mM. Final concentration of PolyA in the phosphorolysis assay was 1.5mM.

— ● — ● — Phosphorolysis μmole/hr
 — □ — □ — Polymerisation μmole/hr

TABLE 2

Substrate specificity of *B.stearothermophilus* PNPase

Phosphorolysis		V (nmole/hr)	
Poly A		36.50	
Poly I		5.00	
Poly C		<0.1	
Poly G		<0.1	
Poly U		<0.1	
Polymerization			
	- Primer μmole/hour	+Primer(Ap) ₄ A	Enhancement
ADP	1.32	1.68	1.27
IDP	1.22	1.41	1.16
GDP	0.035	0.216	6.17
CDP	0.0024	0.720	300
UDP	0.084	0.440	5.23

Reactions were carried out in a total volume of 100 μl as described in the methods section.

prepared by chemical polymerisation and consisted of oligomers with chain lengths from 3 up to 12 residues.

The p-aminophenyl oligo(dT)-Sephadex has been used successfully in the purification of polynucleotide phosphorylase from two different sources. The matrix could be used for the rapid screening of micro-organisms and tissues for the presence of this enzyme.

The marked preference of the enzyme for adenosine (and inosine) residues could be of use in the removal of the polyadenylic acid sequence from the 3'-termini of messenger RNA's. Further studies on the base specificity are in progress.

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