PURIFICATION AND PROPERTIES OF CALF THYMUS POLYADENOSINE DIPHOSPHATE RIBOSE POLYMERASE

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1. Introduction

Polyadenosine diphosphate ribose (poly(A)DPR) polymerase was discovered in our laboratory in chicken liver nuclei in 1966 and the structure of the poly(A)DPR established [1]. The presence of the poly(A)DPR in vivo was demonstrated [2–4]. The existence of the enzyme in rat liver nuclei was reported [5,6]. The enzyme system was also found in plant nuclei [7], some microorganisms [8,9] and *E. coli* phage [10]. Thus, poly(A)DPR polymerase is a ubiquitous nuclear enzyme. In eukaryotic cells, the enzyme is tightly associated with chromatin. Recent studies have indicated that poly(A)DPR polymerase activity was associated primarily with template active regions (euchromatin) whereas the transcriptionally inert chromatin fractions were found to contain relatively low levels of ADP-ribosylating activity [11].

Poly(A)DPR polymerase catalyzes the formation of a homopolymer of ADP-ribose units linked by 1′–2′ glycosidic bonds [2,6]. The substrate for this reaction is NAD^+, and in the presence of DNA, the enzyme successively adds ADPR units onto an initial ADPR residue which may be covalently attached to various nuclear proteins, including histones, non-histone chromosomal proteins, and a Ca^{2+},Mg^{2+}-dependent endonuclease [12–14]. In nuclease-digested chromatin, a considerable portion of poly(A)DPR is intimately associated with the 11 S nucleosome monomer (ν-bodies) [11].

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Although the exact functions of poly(A)DPR polymerase and its product, poly(A)DPR, remain unknown, a variety of evidence suggests its possible role in the modulation of gene replication, repair or expression, as well as in the maintenance of chromatin architecture [15–18]. However, studies on the function of the polymerase have been performed on isolated nuclei or chromatin, and therefore it seems essential to use purified polymerase and acceptor protein to understand the exact function of poly(A)DPR polymerase in vivo. Although poly(A)DPR polymerase has been more or less extensively purified [19–22] relatively few details on the purification methods and on the properties of the polymerase preparations have been published. In a previous study we reported a 540-fold purification of calf thymus poly(A)DPR polymerase and its properties [23]. The study to be described herein provides a relatively simple method for large scale preparations of an electrophoretically homogeneous enzyme from calf thymus gland. Poly(A)DPR polymerase was purified about 3000-fold with a yield of 6%.

2. Materials and methods

[*adenine*-2,8-^{3}H]NAD^+ was purchased from New England Nuclear; cold NAD^+, calf thymus DNAase I and bovine serum albumin from Sigma Chemicals Co. Ultrogel ACA 34 and ampholine were obtained from LKB. Sephadex G-150, Sepharose 4B and blue dextran were purchased from Pharmacia Fine Chemicals. Blue dextran–Sepharose (available from Pharmacia) was prepared according to [24]. Solubilized chromatin

was prepared from calf thymus nuclei according to [25]. Calf thymus gland was removed immediately after killing of animals and stored in liquid nitrogen until used. Enzyme activity is stable at least one year under these conditions. Poly(A)DPR polymerase activity was measured as described [23]. Protein was determined by Lowry's method after precipitation of the protein with trichloroacetic acid to remove interfering substance such as 2-mercaptoethanol [26]. Polyacrylamide–sodium dodecyl sulfate gel electrophoresis was carried out by the method [27]. Isoelectric focusing gels (pH 3–10) containing 7.5% acrylamide were prepared according to [28] and stained according to [29].

3. Results

3.1. *Preparation of nuclei and extraction of polymerase*

About 500 g calf thymus stored in liquid nitrogen was used for a single experiment. The frozen tissues were cut into small pieces, immersed in 3 liter ice-cold 0.25 M sucrose containing 10 mM Tris- HCl (pH 7.9), 10 mM $MgCl_2$, 14 mM 2-mercaptoethanol and 50 mM $Na_2S_2O_5$ (buffer 1). The suspension was homogenized in a mixer (Kenwood) for 2 min at position 1 and 3 min at position 2, and filtered through four layers of cheese-cloth. The filtrate (fraction I) was centrifuged at 9000 rev/min (about 13 200 × *g*) in the GSA head of the Sorvall RC2-B centrifuge for 15 min. The supernatant was discarded and the pellet was washed with buffer 1 (approx. 6 ml buffer 1/g tissue) by mixing for 2–3 min in a mixer at position 1. The solution was centrifuged at 9000 rev/min for 15 min. The washing process is repeated until the supernatant is clear; normally two washings are adequate. The pellet containing the crude nuclei was mixed in approx. 1000 ml buffer 1 and blended in a mixer for 1 min (fraction II). Two hundred ml 2 M NaCl were added in the nuclei suspension and thoroughly homogenized with a spatula for 3 min or until the nuclei suspension becomes a clear transparent solution. At this stage all nuclei are broken and the solution should be very viscous. The solution was centrifuged at 30 000 rev/min (about 100 000 × *g*) in the rotor type 30 for 90 min at 2°C in a Beckman model L2 ultracentrifuge and a quite clear, non-viscous solution was obtained (fraction III). The sticky pellet was discarded. The crude supernatant solution was clarified by filtrating through two layers of cheese-cloth, if necessary. Solid ammonium sulfate (230 g/liter) was added and allowed to stand for 15 min with gentle stirring. This solution was centrifuged at 9000 rev/min for 20 min. The precipitate was discarded. In the supernatant solution solid $(NH_4)_2SO_4$ (350 g/liter) was added and stirred for 15 min and centrifuged at 9000 rev/min for 20 min. The precipitate containing enzyme activity was dissolved in 20 ml buffer 2 containing 0.1 M potassium phosphate buffer (pH 6.8), 0.5 M KCl, 14 mM 2-mercaptoethanol, 0.1 mM dithiothreitol, 10 mM $MgCl_2$, 50 mM $Na_2S_2O_5$, 0.5 M KCl and 25% glycerol (fraction IV). Less than 3% of the total protein and all of the poly(A)DPR polymerase activity was recovered in 90% $(NH_4)_2SO_4$ precipitate. All operations described above were performed at 0–4°C.

3.2. *First gel filtration*

Fraction IV was applied to an Ultrogel ACA 34 column (3.6 × 57 cm) equilibrated with buffer 2, and 7.5 ml fractions were collected with a flow rate of 30 ml/h. The elution of the protein was followed by absorbance at 280 nm and 206 nm by an LKB 2089 Uvicord III. All of the fractions from column chromatography were assayed for enzyme activity as described in Materials and methods. Active fractions, usually eluted between two large ultraviolet adsorbing peaks, were pooled and concentrated in a dialyzing tube with solid sucrose at 0°C (fraction V). At this stage the enzyme activity was stable in buffer 2 for at least 3 weeks at 0°C, or two months at −20°C and one year in liquid nitrogen. Fraction V (20 ml) contained about 400 mg protein and more than 80% of the starting enzyme activities.

3.3. *Hydroxylapatite chromatography*

Two preparations of fraction V (prepared from 1 kg calf thymus) were diluted twice with 0.5 M NaCl containing 14 mM 2-mercaptoethanol and 0.1 mM dithiothreitol, and loaded onto a hydroxylapatite column (2 × 35 cm) equilibrated with 0.5 M NaCl, 0.1 mM dithiothreitol, 14 mM 2-mercaptoethanol and 50 mM NaP_i (sodium phosphate buffer) pH 6.2. The column was then washed successively with each 100 ml of 0.05 M, 0.1 M, 0.2 M and 0.4 M NaP_i. All of the solutions contained 0.5 M NaCl and 0.1 M dithiothreitol and 14 mM 2-mercaptoethanol. During

elution, 7.5 ml fractions were collected. Enzyme activity was exclusively found in 0.4 M NaP_i eluate. The active pool from hydroxylapatite, containing about 110 mg protein and 60% of the starting enzyme activity, was concentrated by solid sucrose as described above (fraction VI). Fraction VI can be stored at 0°C or −20°C for several weeks without loss of enzyme activity.

3.4. *Blue dextran–Sepharose*

About 2 ml of fraction VI were diluted with 2 ml 10 mM Tris–HCl, pH 7.9, containing 50 mM $Na_2S_2O_5$, 14 mM 2-mercaptoethanol, 0.1 mM dithiothreitol and 25% glycerol (v/v), and then passed through a blue dextran–Sepharose (1.2 × 70 cm) equilibrated with buffer 3 containing 0.3 M KCl, and 4 ml fractions were collected. The polymerase appeared in the first peak of this column and was not found in the retarded fractions. The active pool of poly(A)DPR polymerase of blue dextran–Sepharose was concentrated by sucrose (fraction VII). About 50 mg protein were obtained from three columns of Blue dextran–Sepharose. The recovery of polymerase activity was about 30% of the starting enzyme activity and 1000-fold purification was obtained. However, this fraction contained trace amounts of contaminating proteins.

3.5. *Second gel filtration*

About 3 ml concentrated fraction VII were loaded directly onto a Sephadex G-150 column (1.9 × 146 cm) equilibrated with buffer 2, and 1.33 ml fractions were collected. The active fractions of poly(A)DPR polymerase were pooled and used for next column chromatography without concentration.

3.6. *Phosphocellulose absorption and elution*

Active pool from Sephadex G-150 was diluted to 0.24 M KCl with buffer 3 and passed through a column (1.2 × 17 cm) of phosphocellulose (P11, Whatman) equilibrated with buffer 3 containing 0.24 M KCl. The column was washed with 100 ml buffer 3 containing 0.24 M KCl and then eluted with a KCl gradient from 0.24–1.24 M in buffer 3. The total volume of the gradient was 200 ml. The polymerase activity was eluted at 0.52 M KCl concentration in buffer 3. The active pool of poly(A)DPR polymerase was concentrated as described above. Elution with this gradient effected the complete elimination of contaminating proteins in fraction VII. The specific activity of the poly(A)DPR polymerase was constant across the entire peak, indicating a high degree of purity. The results of a typical purification from 1 kg calf thymus is shown in table 1.

3.7. *Storage and stability of purified enzyme preparation*

The active pool from phosphocellulose had a specific activity of about 1090 units/mg protein under the condition described above and could be stored at −20°C without loss of activity for several months in buffer 2 or buffer 3 containing 0.5 M KCl.

Table 1
Purification of calf thymus poly(A)DPR polymerase

Procedure		Total activity (units)	Total protein (mg)	Specific activity (units/mg prot.)	Yield (%)	Purification (-fold)
Fr I	Total	57 680	163 770	0.35	(100)	1
Fr II	Nuclei	66 500	113 313	0.59	–	2
Fr III	NaCl Extract	52 349	12 203	4.29	91	12
Fr IV	$(NH_4)_2SO_4$	54 559	4730	11.53	95	33
Fr V	Ultrogel ACA 34	46 920	392	119.5	81	341
Fr VI	Hydroxylapatite	32 701	111	293.8	57	839
Fr VII	Blue dextran–Sepharose	18 189	51	356.6	32	1020
Fr VIII	Phosphocellulose	3437	3.15	1089	6	3111

The enzyme was isolated from 1 kg calf thymus

Activity was assayed and units defined as described in Materials and methods

4. Discussion

4.1. *Fractionation: solubilization of polymerase activity*

The tight association of poly(A)DPR polymerase activities with chromatin has been reported by many workers [1,19,20]. In fact, the first activity detected in the chicken liver was isolated with particulate fractions of nuclear extracts [1]. The solubilized enzyme fraction was obtained by DNAase treatment [19], NaCl extraction and centrifugation [30], CsCl gradient centrifugation [20], and $(NH_4)_2SO_4$ extraction [23]. We have examined DNAase treatment and NaCl extraction to solubilize poly(A)DPR polymerase. Essentially the same results were obtained. However, using DNAase treatment, we have not been successful in finding conditions for solubilization of the enzyme without loss of activity unless highly purified DNAase is used, indicating that the polymerase is highly sensitive to proteolytic enzymes present in several lots of DNAase.

The protection of the enzyme from proteolytic degradation is one of the major problems for poly(A)DPR polymerase purification from calf thymus gland. Only in the presence of high concentration of SH-reagents and sodium pyrosulfite, the enzyme could be purified and was stable for several weeks at 4°C. Relatively high concentration of KCl or NaCl was required to keep the enzyme in soluble state. $MgCl_2$ and glycerol were also added even though they are not absolutely required, but both of them appeared to be necessary for complete protection during enzyme purification. Phenylmethylsulfonyl fluoride was not effective to inactivate the proteolytic enzymes completely, while poly(A)DPR polymerase was insensitive to this protease inhibitor.

4.2. *Purity of enzyme: gel electrophoresis*

A single protein band was found after gel electrophoresis of the phosphocellulose fraction at pH 8.9 and pH 4.5 in the presence of 0.3 NaCl (results not shown). Gels run in the absence of NaCl showed some aggregated material on the top of gels and no protein band had migrated into gels. Electrophoresis of the purified enzyme preparations in 0.1% sodium dodecyl sulfate on 10% acrylamide gels, at pH 8.8, showed a single band of protein as shown in fig.1. Arrow indicates the front of migration determined by bromophenol blue.

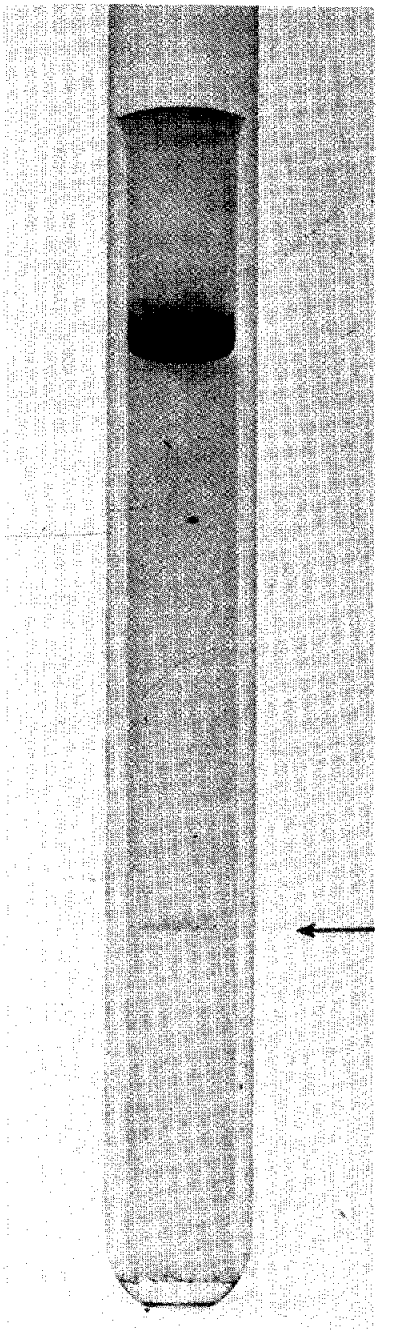

Fig.1. Resolution of the purified poly(A)DPR polymerase by gel electrophoresis on a 10% polyacrylamide gel (pH 8.8) containing 0.1% dodecyl sulfate.

The distances of migration of a number of proteins and purified polymerase in the sodium dodecyl sulfate acrylamide are plotted against molecular weight of the marker proteins. The polypeptide in the calf thymus poly(A)DPR polymerase preparation is estimated to have mol. wt 120 000. This value is in good agreement with the molecular weight estimation from Sephadex G-100 and Ultrogel ACA 34 gel filtration.

A single protein band was found after isoelectric focusing of the concentrated phosphocellulose fraction (results not shown). The polypeptide in the calf thymus poly(A)DPR polymerase preparation is estimated to have a pH_i of 6.5, when the gel is sliced, extracted, and examined for pH.

4.3. *Reaction characteristics (nucleic acid contamination)*

Many workers, including our last publication, reported the absolute requirement of DNA and histone or chromatin for full activity of poly(A)DPR polymerase from rat liver, calf thymus and cultured cells. Our purified enzyme preparation, which still contains traces of nucleic acids as judged from the $A_{280/260\,nm}$ ratio, did not require addition of chromatin or DNA for full activity, although high molecular weight poly(A)DPR is synthetized. Several workers reported the presence of DNA in the purified preparation of poly(A)DPR polymerase, even though the amount of DNA present in the enzyme preparation is quite low [22].

4.4. *Effect of NAD^+ concentration on rate of polymerization (kinetics)*

The purified enzyme was incubated with different concentrations of NAD^+. An apparent K_m of 82 μM was obtained from double reciprocal plot of NAD^+ concentration and enzymatic activity as shown in fig.2. This value is in good agreement with a K_m value obtained from partially purified poly(A)DPR polymerase from calf thymus [23].

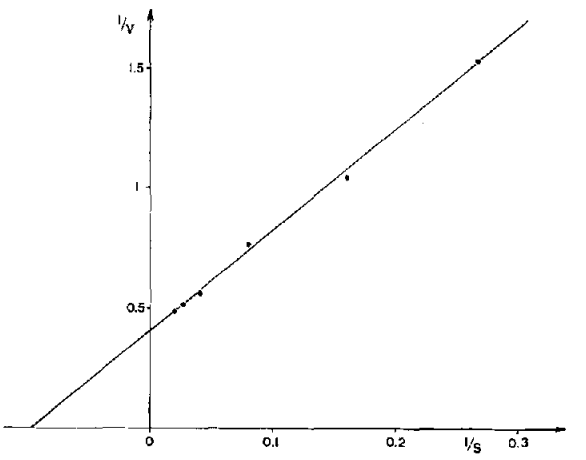

Fig.2. Lineweaver-Burk plot for calf thymus poly(A)DPR polymerase. The standard assay was used with varying amounts of [^{3}H]NAD. K_m 82 μM.

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