A CONVENIENT METHOD FOR THE PREPARATION OF PRIMER-DEPENDENT POLYNUCLEOTIDE PHOSPHORYLASE FROM MICROCOCCUS LYSODEIKTICUS

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Introduction

During the past few years primer-dependent PNPase [1] has been used extensively for the synthesis of polyribonucleotides with a known sequence at the 5′-hydroxyl end (Leder et al., 1965; Thach, 1966; Stanley et al., 1966). Primer-dependent preparations of PNPase from M. lysodeikticus (Singer and Guss, 1962; Singer and O'Brien, 1963; Thanassi and Singer, 1966) are particularly useful for these syntheses. During polymerization the enzyme catalyzes the elongation of preformed oligonucleotides, which thereby become the 5′-hydroxyl ends of the new chains. Recently, Klee (1967) reported that the original procedures no longer consistently yield primer-dependent enzyme. The form of PNPase (namely, primer-independent or primer-dependent) obtained in a given preparation appears to depend on the particular batch of cells used (all cells are obtained commercially from Miles Laboratories). Klee (1967) also reported several chemical procedures whereby primer-independent enzyme could be made primer-dependent. One of these, treatment with trypsin, has proven useful on a preparative scale and we report here the details of this method. The convenience of the procedure is enhanced by the use of crude, commercially available enzyme as the starting material.

Preparation of Primer-Dependent PNPase

All procedures were carried out at 0-4° unless specifically noted otherwise. The source of all the materials as well as the enzyme assays have

[1] Abbreviations used are as follows: Polynucleotide phosphorylase, PNPase; oligonucleotides and mononucleotides are abbreviated according to the scheme described in the Journal of Biological Chemistry; bovine serum albumin, BSA.

been described in detail previously (Singer and O'Brien, 1963; Thanassi and Singer, 1966). The results of the purification are summarized in Table I. Each fraction may be stored frozen at -20° prior to proceeding to the next step.

TABLE I. Preparation of primer-dependent PNPase

	Fraction	Protein	$\frac{A_{280}}{A_{260}}$	Phosphorolysis[a,b]		Polymerization[a,c]	
				Specific activity	Total units	Specific activity	Total units
		mg/ml		units/mg		units/mg	
I.	Crude enzyme	31.5	1.6	0.3	19.9	4.1	282
II.	Pooled G-200	0.3	1.3	1.7	16.7	22.5	215
III.	Trypsin treated	1.5	1.3	3.7	9.3	31.2	78
IV.	Pooled G-200	1.9	1.5	5.6	7.2	52.5	67

[a] Specific activity defined as units per mg protein.

[b] One phosphorolysis unit is equivalent to the formation of 1 μmole of ADP from poly A under the standard conditions (Singer and Guss, 1962).

[c] Polymerization was measured in the presence of 6.8 A_{257} units of ApApApA per ml. One unit is equivalent to the polymerization of 1 μmole of ADP in 15 min under the standard conditions.

The starting material for this preparation was 100 mg of lyophilized M. lysodeikticus PNPase obtained from P-L Biochemicals, Inc. It was dissolved in 0.01 M Tris-HCl, pH 8.2, containing 1 mM EDTA, 1 mM β-mercaptoethanol and 0.1 M NaCl (Buffer A) to yield a final volume of 2.8 ml (Fraction 1). A portion of the solution (2.2 ml) was applied to a column of Sephadex G-200 (2.5 x 35 cm) that had previously been equilibrated with Buffer A. The column was eluted with the same buffer at a flow rate of 0.25 ml per min and 3.2-ml fractions were collected. The fractions were assayed for their ability to polymerize ADP in the presence and absence of primer, in this case, ApApApA, and their absorbancy at 280 mμ was determined. The results are shown in Fig. 1A. The activity emerges at the void volume of the column and is thereby separated from the bulk of the protein. Polymerization is stimulated about 2-fold by the addition of ApApApA. The fractions collected between 44 and 73 ml of eluting fluid were pooled (Fraction II, 29 ml). Fraction II was

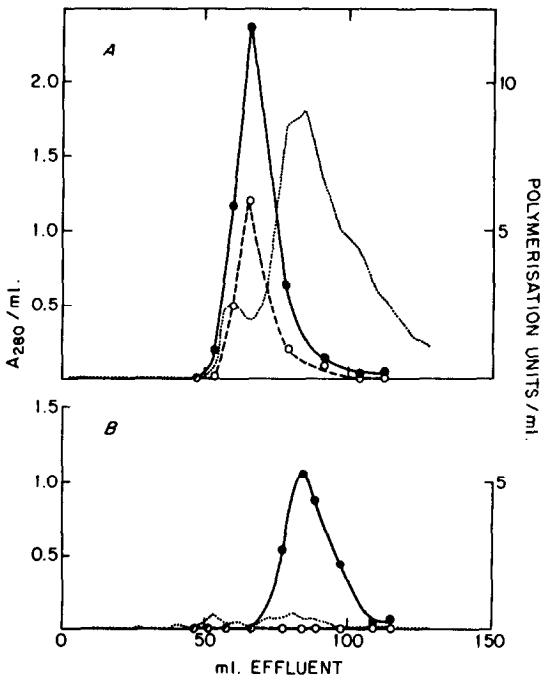

FIG. 1. Purification of M. lysodeikticus PNPase by filtration through Sephadex G-200 before and after treatment with trypsin.

1A. Fraction I (Table I) was passed through the column as described in the text. The abscissa records the ml of column effluent. A_{280}, ...; polymerization units per ml in absence --o--o and in presence —●—, of ApApApA (see Table I).

1B. Fraction III (Table I) was passed through the same column used for the experiment in Fig. 1A as described in the text. The symbols are as defined for Fig. 1A.

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treated with trypsin as follows: 392 μg of trypsin (Worthington, 2 times crystallized), freshly dissolved in 1.12 ml of H_2O, were added to 28 ml of Fraction II, previously brought to room temperature. After incubation for 2 hours at room temperature, 0.56 ml of 5 x 10^{-3} M diisopropylfluorophosphate (Mann) was added to stop the reaction. The solution was placed in a collodion bag (Schleicher and Schuell) and concentrated under vacuum to 1.7 ml (Fraction III). The major portion of Fraction III (1.5 ml) was applied to the same Sephadex G-200 column used earlier. The column had been washed extensively with Buffer A modified to contain 0.2 M NaCl; enzyme was eluted with this

buffer (0.1 M NaCl is equally suitable when larger amounts of protein are used). Fractions of approximately 2.3 ml were collected and assayed as before (Fig. 1B). As a result of the trypsin treatment, the activity now emerges from the column well after the void volume, suggesting that the enzyme is modified. The mobility of the modified enzyme is not affected by the NaCl concentration in the range 0.1 to 0.2 M. No activity was detected in the absence of primer. The material eluted between 68 and 91 ml after the start of the column was combined (23 ml) and concentrated as described above to less than 0.3 ml. Buffer A containing 0.2 M NaCl was used to wash it out. The final solution (Fraction IV, 0.7 ml) was stored frozen.

This procedure results in the recovery of about 35% of the enzyme units (Table I). Considerably more than 100 mg of crude enzyme can be worked up by appropriate increase in the size of the first Sephadex column. The loss of activity accompanying the trypsin treatment has been repeatedly observed: a greater fraction of polymerization units compared to phosphorolysis units is invariably lost during this treatment. The decrease in activity occurs during the first 30 min of trypsin treatment and no further decrease is observed after 2 hours. Small-scale trials of the trypsin digestion were followed by measurement of polymerization in the presence and absence of primer as well as by disc gel electrophoresis. It was recently demonstrated (Klee, 1967) that both primer-dependent and -independent enzymes have completely different electrophoretic mobilities. By this criterion, Fraction I was mainly the primer-independent species but contained some of the primer-dependent form while Fraction II was essentially the primer-independent species. Two hours of incubation with trypsin were required to convert all of Fraction II to primer-dependent form. It is advisable to perform preliminary trypsin digestions on a small scale for each preparation in order to ascertain conditions required for complete conversion to primer-dependent form.

Fig. 2 demonstrates the primer-dependence of Fraction IV. Even in the absence of primer, polymerization does occur after a lag period at high enzyme

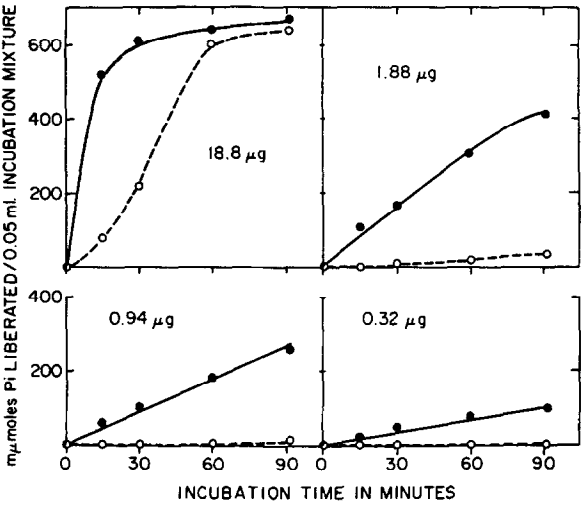

FIG. 2. The dependence of ADP polymerization on oligonucleotide primer as a function of enzyme concentration. All reaction mixtures (0.3 ml) contained 0.1 M Tris-HCl, pH 9.0, 0.4 mM EDTA, 5 mM $MgCl_2$, 0.02 M ADP, and 60 μg BSA. The oligonucleotide ApApApA served as primer and was added, where indicated, in a concentration of 9.0 A_{257} per ml. Incubation was at 37°. At the indicated times, 0.05 ml aliquots were removed and added to 0.95 ml of 2.5% $HClO_4$. After centrifuging, Pi was determined on suitable aliquots by the method of Fiske and SubbaRow. The ordinate records the mμmoles of Pi liberated in the 0.05 ml aliquot: •—•, plus primer; o---o, minus primer.

concentrations (Thanassi and Singer, 1966). The final extent of the reaction is the same in the presence or absence of added primer. The data (Fig. 2) indicate that ApApApA, under the conditions used, stimulates the initial rate of ADP polymerization about 20-fold. Polymerization of either UDP or CDP is similarly dependent on oligonucleotide. As expected from earlier work (Singer and Guss, 1962; Singer and O'Brien, 1963), an oligonucleotide is inactive as primer if its terminal C-3′ hydroxyl group is phosphorylated; active oligonucleotide primer is incorporated into the newly formed polymer (Table II).

Fraction IV was tested for nuclease activity under the conditions of the polymerization reaction. ^{3}H-Poly A (6.2 μmoles per ml) was incubated in the presence of 20 polymerization units per ml of Fraction IV. In 210 min at 37°, none of the ^{3}H had been made soluble in 2.5% $HClO_4$. The solubilization of 0.2 μmole of poly A per ml of reaction mixture would have been detected

easily. Thus, nuclease activity was less than 0.1% of the polymerization activity.

TABLE II. The incorporation of ^{14}C-pApApApA into poly A

Time of incubation	cpm Recovered as	
	pApApApA	$(pA)_n$, n > 4
min		
0	1596	28
12	707	862
24	431	1091
36	155	1292
48	198	1408
48 (no enzyme)	1507	15

Reaction mixtures (0.05 ml) contained 0.1 M Tris-HCl, pH 9.0, 0.4 mM EDTA, 5 mM $MgCl_2$, 0.02 M ADP, 8 μg BSA, 0.42 A_{260} units of ^{14}C-pApApApA (Nossal and Singer, in preparation) (containing 2236 cpm) and 3.8 μg of Fraction IV. At the indicated times 1 μmole of EDTA was added to stop the reaction. The reaction mixtures were spotted on Whatman DEAE chromatography paper (DE-81) and chromatographed for 6.5 hr with 1 M NH_4HCO_3, at room temperature. The ADP and pApApApA of the zero time, which were visible in ultraviolet light, served as markers. The paper strips were cut into 1 cm bands and counted in a toluene scintillation fluid. All counts in the area corresponding to pApApApA (R_f = 0.35) were added up. All counts corresponding to R_f values less than 0.35, including the origin, were considered to be incorporated into polymer. There was no significant radioactivity found in areas with Rf greater than 0.35. The R_f of ADP was 0.69.

REFERENCES

Klee, C. B., J. Biol. Chem., 242, 3579 (1967).
Leder, P., M. F. Singer, and R. L. C. Brimacombe, Biochemistry, 4, 1561 (1965).
Singer, M. F., and J. K. Guss, J. Biol. Chem., 237, 182 (1962).
Singer, M. F., and B. M. O'Brien, J. Biol. Chem., 238, 328 (1963).
Stanley, W. M., Jr., M. A. Smith, M. B. Hille, and J. A. Last, Cold Spring Harbor Symp. Quant. Biol., 31, 99 (1966).
Thach, R. E., in G. L. Cantoni and D. R. Davies (Editors), Procedures in Nucleic Acid Research, Harper and Row, New York, 1966, p. 520.
Thanassi, N. M., and M. F. Singer, J. Biol. Chem., 241, 3639 (1966).