THE SOLUBILIZATION AND PARTIAL CHARACTERIZATION OF PEA RNA POLYMERASES

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SUMMARY

A technique has been developed for solubilizing RNA polymerase from pea seedling, which employs the sonication of frozen cells and the addition of insoluble polyvinylpyrrolidone(Polyclar AT) to prevent inactivation of the enzyme. RNA polymerases from peas have properties similar to those of other eucaryotes.

INTRODUCTION

Recent studies of RNA polymerase and its role in bacterial and phage transcription(1-4) have stimulated the study of its role in eucaryotic cells. Multiple forms of RNA polymerases are found in nearly every eucaryotic organism (5-9). In higher plants, however, RNA polymerases have been partially purified only from maize(10-12), wheat(13,14), coconut nuclei(15-17) and from soybean chromatin(18). Their properties are similar to RNA polymerases from animal cells. Biochemical studies of plant growth regulators(19-21) and their photic effects on plant tissues prompted us to study this RNA polymerase. The extraction methods previously reported(10-17) were not applicable to the solubilization of pea enzymes, but disruption by sonication followed by the addition of Polyclar AT to remove phenolics is effective for producing multiple RNA polymerases. The enzymatic properties of these enzymes and their sensitivities to drugs are also discussed.

MATERIAL AND METHODS

Chemicals Highly-polymerized calf thymus DNA, CTP, UTP and GTP were

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obtained from Sigma Chemical Co.. Rifamycin and α-amanitin were purchased from Calbiochem. 3H-UTP was obtained from the Radiochemical Center; ATP from Kojin Chemical Co.; and Polyclar AT from the General Aniline and Film Corp.

Growth of pea seedling Seedling of Alaska pea(*Pisum sativum* var. Alaska) were grown in darkness at 25° for 6 days, then were exposed to light for 24 hr under semi-sterile conditions. After 24 hr of light exposure, plants became green. Apical buds, 10mm long, were harvested and frozen until use.

Preparation of DNA-dependent RNA polymerase Twenty grams of frozen apical bud was ground in a mortar for 10 min in a final volume of 40 ml containing 0.15M tris-HCl buffer, pH 7.8(at 25°); 0.1mM EDTA; 5mM $MgCl_2$; 5mM dithiothreitol (DTT); 0.01M ammonium sulfate and 25% v/v glycerol. The mixture was sonicated for 2 min, then 20 g of washed Polyclar AT(22) was immediately added. The homogenate was filtered through four layers of cheesecloth and centrifuged at 100,000 X g for 60 min in a Type 50 rotar of a Spinco preparative ultracentrifuge. The supernatant was dialyzed overnight against 50mM tris-HCl buffer, pH 7.5; 0.1mM EDTA; 0.5mM $MgCl_2$; 1mM DTT; 0.01M ammonium sulfate and 25% glycerol(0.01M ammonium sulfate standard buffer).

Assay for RNA polymerase activity Enzyme activity was defined as the amount of 3H-UMP incorporated into 5% CCl_3COOH insoluble material counted on filter paper discs after applying 100μl of the reaction mixture as previously reported (9). The standard reaction mixture contained in 0.15 ml: 50mM tris-HCl buffer, pH 7.8(at 25°); 2mM $MnCl_2$; 2mM DTT; 0.32mM of ATP; GTP; CTP; 0.05mM of 3H-UTP (36 counts X min^{-1} X $pmol^{-1}$); 12 mg of native or denatured calf thymus DNA; and 50μl of enzyme solution. Incubation lasted for 10 min at 37°. The reaction was started by adding the enzyme fraction and was terminated by pipetting the reaction mixture onto a 2.3 cm disc(Whatmann 3MM). Then, after 1 min, it was put into 5% CCl_3COOH containing 0.01M sodium pyrophosphate. The disc was washed three times with 5% CCl_3COOH containing 0.01M pyrophosphate, then twice with acetone to remove water. After drying, the radioactivity of the disc was counted in scintillation fluid with a Packard Scintillation Spectrophotometer.

RESULT

High-speed supernatants obtained from the three extraction methods used showed almost no incorporation of UMP. On DEAE-Sephadex chromatography, however, these crude extracts gave one or two activity peaks. Only one peak emerging at 0.20-0.24M ammonium sulfate (which prefers the denatured template) was observed in the elution profile of the crude extract obtained from ground frozen cells (Fig 1, A). When sonication was employed after grinding, the chromatogram gave a very slight peak at 0.20-0.24M. Another peak appeared at 0.1M ammonium sulfate, which had almost the same activity for native and denatured templates (Fig 1, B). This shows that sonication is effective for extracting the lower salt peak. On adding a phenolic absorbent, Polyclar AT, to the sonicated mixture, both peaks were found(Fig 1, C). The size of these peaks was independent of the salt concentration of the homogenizing solution,(0.01M and 0.3M), although different results were obtained with animal cells(23). We designated these enzymes, in order of their elution, enzymes I and II. The pooled fraction of each peak was precipitated by ammonium sulfate and was used in the characterization experiment after dialyzing the enzyme solution against buffer containing: 50mM tris-HCl, pH 7.5; 1mM DTT; and 25% glycerol. The specific activities of enzyme I and II were 100 and 600 pmol of UMP, respectively, incorporated per mg of protein per 10 min at 37°. Preparations were active for several weeks when stored at -20° in 25% glycerol. The RNA synthesis catalyzed by each of these peaks is completely dependent upon added DNA.

The divalent cation requirement of each enzyme shown in Fig 2 indicates that enzyme II prefers Mn^{++} to Mg^{++}; but with enzyme I almost the same activity peak was observed at the optimum concentrations of both Mn^{++} and Mg^{++}. The curves for enzyme I activity against the concentration of Mg^{++} displayed a broad optimum at 10 to 20mM, whereas Mn^{++} had its maximum activity at 2mM.

Salt dependency curves for each enzyme's activity in the presence of 2mM Mn^{++} or 15mM Mg^{++} show that the optimum concentration is at 0.10M ammonium sulfate (see Fig 3) for both enzymes in the presence of Mn^{++}. In the presence of Mg^{++}

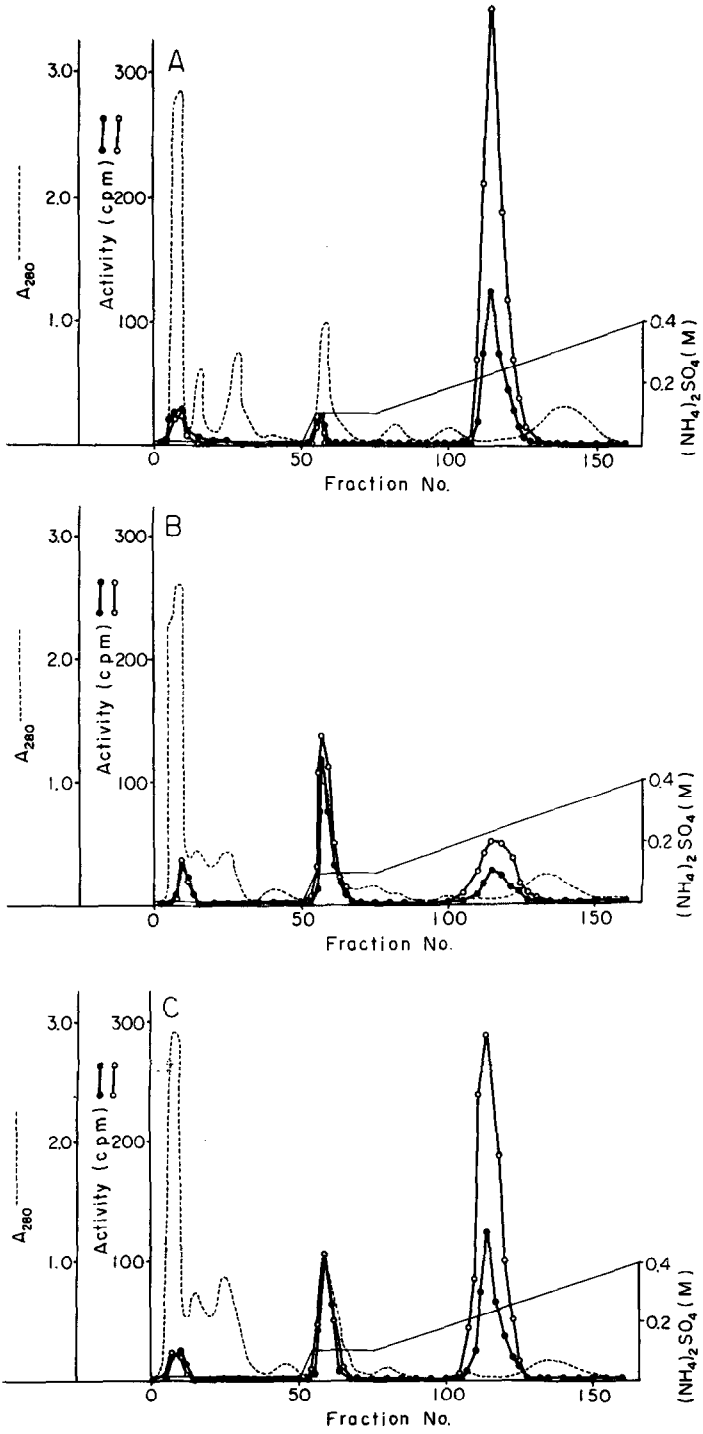
A
B
C
A_{280}
Activity (cpm)
$(NH_4)_2SO_4$ (M)
Fraction No.
3.0
2.0
1.0
300
200
100
0.4
0.2
0
50
100
150

Fig. 1.

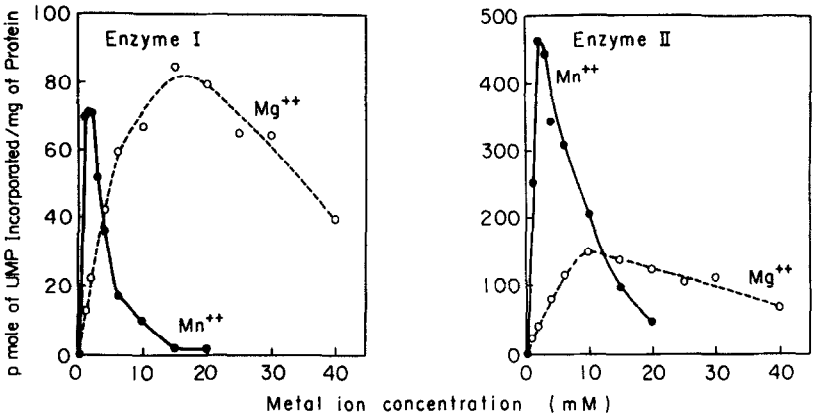

Fig 2 Effect of Mn^{++} and Mg^{++} on RNA polymerase activity. Assay conditions were as described in Methods except for metal-ion concentrations. Ammonium sulfate was not included in the enzyme preparation. Protein was determined by Lowry's method(24) using bovine serum alubumin as standard. Incubation was at 37° for 10 min. The native template was used.

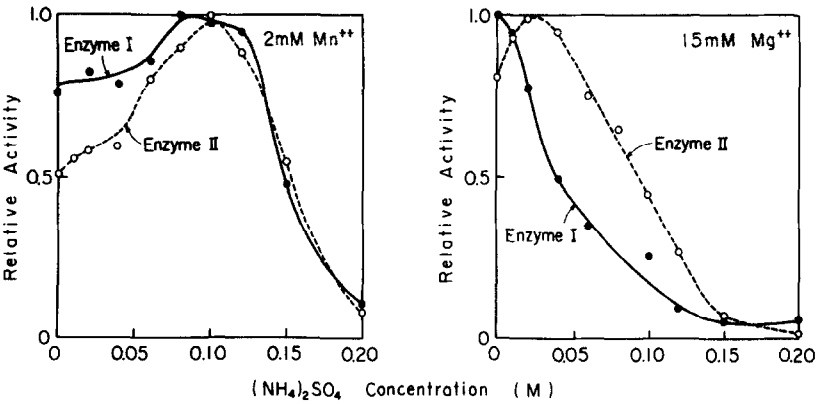

Fig 3 Effect of ionic strength on RNA polymerase. Reaction conditions were as described in Methods except that the ammonium sulfate concentration was varied as indicated. The metal-ion concentration was 2mM for Mn^{++} and 15 mM for Mg^{++}. The native template was used.

Fig 1 DEAE-Sephadex chromatography of RNA polymerase.
15-17 ml of crude extract from 10 g of pea buds was loaded on a 1.5 X 25 cm DEAE-Sephadex(A-25) column equilibrated with 0.01M ammonium sulfate standard buffer. After loading, the column was washed with 150 ml of the above buffer and eluted with 75 ml of 0.1M ammonium sulfate standard buffer, then with 280ml of a linear gradient of 0.1-0.4M ammonium sulfate standard buffer. A flow rate of 24ml/hr was used. 3ml fractions were collected and assayed as described in Methods. Incubation was at 37° for 15 min. (A) Crude extract was obtained only by grinding; (B) Crude extract was obtained by sonication after grinding; (C) Crude extract was obtained by the addition of Polyclar AT to the sonicated mixture; (●——●) Activity with native template; (o——o) Activity with denatured template.

Table 1 Sensitivity of enzyme I and II to drugs

The incubation system was the same as described in Methods except that the ammonium sulfate concentration was 0.08M. The native template was used. Rifamycin was dissolved in 40% v/v glycerol solution.

Drugs	Concentration µg/ml	Enzyme I		Enzyme II	
		Activity (cpm)	Inhibition (%)	Activity (cpm)	Inhibition (%)
Control	-	167	-	386	-
Rifamycin	63.0	175	0	400	0
α-Amanitin	0.13	142	15	27	93
	0.65	130	23	5	99
	1.3	137	18	10	98

ammonium sulfate had an inhibitory effect on enzyme I. For enzyme II, 0.02M is the optimum concentration.

Sensitivity to drugs is shown in Table 1. The mushroom toxin, α-amanitin, inhibited enzyme II almost completely, but had no significant inhibitory effect on enzyme I. Rifamycin, an inhibitor of bacterial RNA polymerases, had no inhibitory effects on either enzyme.

DISCUSSION

Two DNA-dependent RNA polymerases characteristic of eucaryotic cells were isolated from pea buds. Enzyme II was extracted by only grinding the frozen cell, whereas sonication was required to solubilize enzyme I. Unfortunately, sonication resulted in the inactivation of enzyme II, presumably due to the presence of solubilized phenolics. Polyclar AT treatment of sonicated buds was useful for extracting the two forms. Polyclar AT is an effective absorbent for phenolics and is easily separated from the enzyme solution by centrifugation(22). Our method is useful for investigating decreases and increases in the amounts of enzyme, or the appearance of new enzymes which might occur due to changes in enviromental conditions at some stage of development, because it avoids variations in the amounts of nuclei recovered from the isolation procedure.

From their template preferences and sensitivities to α-amanitin, enzyme I seems to be nucleolar and enzyme II nucleoplasmic(5). Since the RNA polymerases, extracted from etiolated pea seedlings which have no chloroplasts, gave the same profile on DEAE-Sephadex, it is unlikely that these two peaks originate from chloroplasts.

The cation requirement is analogous to that for animal enzymes; enzyme I prefers Mg^{++} and Mn^{++}, while enzyme II prefers Mn^{++} to Mg^{++}. The activity of enzyme II is completely inhibited by α-amanitin, as in animal cells. Enzyme I is partly inhibited by α-amanitin, whereas enzymes from animal cells are completely resistant to α-amanitin. This result was also obtained with two other higher plants; maize(11) and coconut(17), and may be a general property of plant enzymes. Insensitivity to rifamycin excludes the possibility of bacterial contaminations.

The absence of activity in the crude extract and the appearance of activity during chromatography suggests the presence of an inhibitor. The characterization and purification of this inhibitor is now in progress.

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