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SOLUBLE AND CHROMATIN-BOUND DNA POLYMERASES IN DEVELOPING SOYBEAN

M. M. D'Alesandro, R. H. Jaskot, and V. L. Dunham

Department of Biology, State University College
Fredonia, New York 14063

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Summary: Soluble and chromatin-bound DNA polymerases were isolated and partially purified from germinating embryos and 96-hour, etiolated soybean hypocotyls. The homogenization buffer contained 1.2 M sucrose and 30% glycerol to maximize nuclear integrity. Although the specific activity of the chromatin-bound enzyme increased 6-fold during the first 48 hours of germination, most of the polymerase activity (units/g dry wt.) was in the soluble fraction. Observed developmental changes in the polymerase activities were characterized by changes in sensitivity to KCl and Mg^{2+}. Specific assay conditions indicated that the soluble and chromatin-bound fractions had similar characteristics to DNA polymerase α and β, respectively.

Plant DNA polymerases isolated in aqueous buffer have been identified as soluble (1-4) and chromatin-bound (5-8). Total chromatin-bound activity remains relatively constant during early seed germination, whereas the soluble enzymes display a characteristic increase in activity (9-11). Similar changes in polymerase activity during active cell proliferation have been observed in animal systems in which DNA polymerase-α increases in activity and DNA polymerase-β remains constant (12).

The purpose of this initial study was to determine the amount of soluble and chromatin-bound DNA polymerase activity in the early stages of seed germination by employing an homogenization buffer which would maximize nuclear integrity (13). The investigation identified changes in the characteristics and types of polymerases during the first four days of growth and development.

Materials and Methods: Soybean seeds (*Glycine max*, var. Harosoy-63) were rinsed in tap water and 0.05% $HClO_3$ for ten minutes. Following extensive rinsing with glass distilled water, seeds for germination studies were soaked for an additional two hours and then layered between sterilized, moistened, cheesecloth. After various times in the dark at 29°C, the plants were harvested and the entire embryonic axis was used as a source of enzyme. The seeds which were used as a source of 96-hour DNA polymerase activity, were planted in moist vermiculite. Seedlings were grown in the dark at 29°C for 96 hours. The hook region of the hypocotyl was the source of enzyme.

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The embryos were homogenized in a buffer containing 50 mM Tris-HCl (pH 7.5); 1.2 M sucrose; 20 mM $MgCl_2$; 10 mM 2-mercaptoethanol; 20 mM KCl and 30% glycerol in a ratio of 1.5:1 (volume/fresh weight). Following homogenization for a total of one minute at setting 5 with a Polytron PCU-2, the crude homogenate was filtered through cheesecloth and Miracloth and centrifuged for 40 minutes at 20,000 x g. The resulting nuclear pellet was scraped from the underlying starch layer and resuspended in a 50 mM Tris buffer (pH 7.5) containing 5% glycerol; 5 mM $MgCl_2$; 0.1 mM EDTA and 0.5 mM 2-mercaptoethanol. The resuspended nuclear pellet was sonicated with a Fisher sonicator (Model 300, setting 75%). The sonicate was stirred in the cold for two hours and centrifuged at 100,000 x g for 60 minutes. The supernatant, containing the enzyme solubilized from chromatin, was desalted through a Sephadex G-50 column before use. The original supernatant was dialyzed in the cold against a 0.02 M KPO_4 buffer (pH 7.5) containing 0.1 mM EDTA and 5% glycerol and then centrifuged at 20,000 x g for 30 minutes. The supernatant was used as a source of soluble enzyme.

Tissue for the 96-hour enzyme study was homogenized as described in the germination study. The nuclear enzyme was extracted from the initial pellet using procedures described for 48-hour embryos. The supernatant, following homogenization, was subjected to ethanol precipitation using 95% ethanol to a final concentration of 74%. The slurry was stirred in the cold and centrifuged at 20,000 x g for 30 minutes. The pellet was resuspended with the dialysis buffer used in the germination study and sonicated. The sonicate was dialysed in the cold against the 0.02 M KPO_4 buffer (pH 7.5) and centrifuged at 20,000 x g for 40 minutes. The resulting supernatant was used as the 96-hour soluble enzyme.

The soluble and nuclear preparations extracted from the hypocotyls were eluted from separate hydroxylapatite columns (0.75 x 6.0 cm) at 0.15 M KPO_4 (15- to 30-fold purification). All assays for total DNA polymerase activity were previously described (8). Assays to determine the presence of the various types of DNA polymerases were as follows: α-assay: 20 mM potassium phosphate (pH 7.5); 8 mM $MgCl_2$; 1.0 mM 2-mercaptoethanol; 0.1 mM EDTA; 0.05 mM dNTP's; 0.005 mCi ^{3}H-TTP (specific activity 80 mCi/μmole); 20 μg DNA and 0.05 ml enzyme (40-100 μg protein) in a total volume of 0.4 ml, β-assay: 50 mM Tris-HCl (pH 8.3); 8 mM $MgCl_2$; 100 mM NaCl; 1.0 mM 2-mercaptoethanol; 5.0 mM N-ethylmaleimide; 0.05 mM dNTP's; 0.005 mCi ^{3}H-TTP; 20 μg DNA and 0.05 ml enzyme (40-100 μg protein) in a total volume of 0.4 ml.

γ-assay: 20 mM Tris-HCl (pH 8.3); 50 mM potassium phosphate (pH 8.4); 0.5 mM $MnCl_2$; 100 mM KCl; 2.0 mM dithioerythritol; 40 μg BSA; poly (A)·(dT) 12-18 (50 μmoles/ml); 0.005 mCi ^{3}H-TTP and 0.05 ml of enzyme (40-100 μg protein) in a total volume of 0.4 ml (14). Protein determination was by the Lowry method (15). Data presented are averages of a minimum of three separate experiments.

Results: The homogenization of the tissue in sucrose and glycerol reduced nuclear breakage as evidenced by the presence of intact nuclei in the crude homogenate. The total enzyme units in this soluble fraction increased after six hours following the onset of germination (Fig. 1A). By 48 hours, the total units were 8-fold greater than at 6 hours and 16-fold greater than that of the chromatin fraction. The amount of chromatin-bound enzyme increased slightly (1-fold) by 48 hours. The specific activity increased in the chromatin fraction after 24 hours (Fig. 1B). Although specific activity in the soluble fraction exhibited a slight increase from the onset of germination, by 48 hours it was

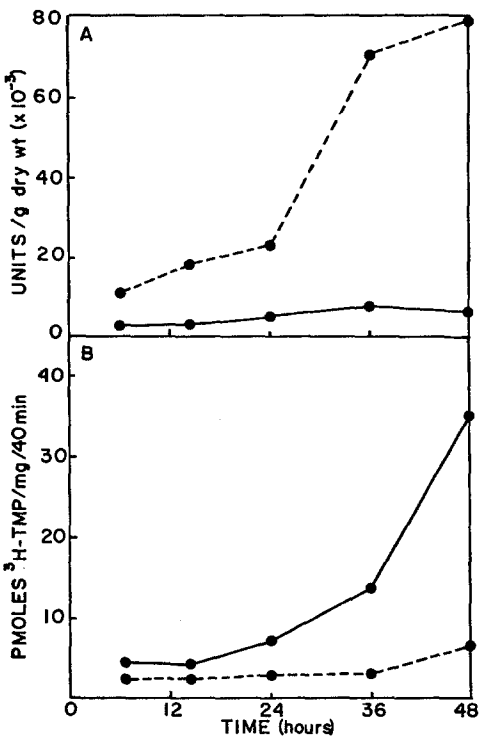


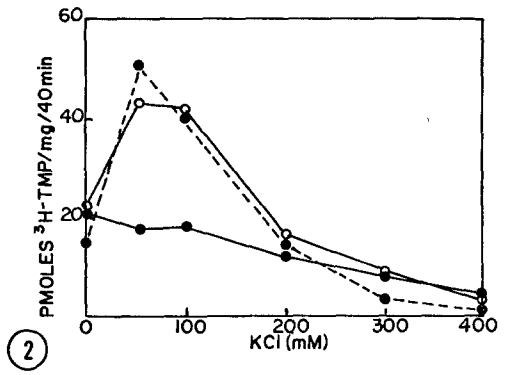
Figure 1A. Increase of DNA polymerase activity during seed germination ●—● chromatin-bound activity; ●---● soluble activity.

1B. Specific activity of DNA polymerase during seed germination ●—● chromatin-bound activity; ●---● soluble activity.

five times less than that of the chromatin enzyme. The majority of the total enzyme units were associated with the soluble fraction but the chromatin enzyme exhibited a much higher specific activity.

A comparative analysis of the 48-hour soluble and both 96-hour enzymes (chromatin and soluble) indicated the requirement for DNA and all four deoxyribonucleoside triphosphates for maximum activity. Deletion of 2-mercaptoethanol had no effect on enzyme activity. The soluble 48-hour enzyme showed no requirement for divalent cations (Mg^{2+} or Mn^{2+}). Both 96-hour fractions, however, exhibited a strong dependence on Mg^{2+} (optimum activity at 10 mM) which could not be compensated for with Mn^{2+}.

Low concentrations of KCl (50-100 mM) stimulated the chromatin and soluble 96-hour enzyme 2- and 3-fold respectively (Fig. 2). However, all concentrations of KCl inhibited the 48-hour soluble activity.



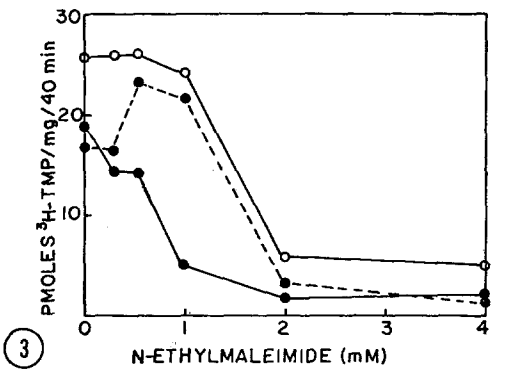


Figure 2. Effect of KCl on DNA polymerase activity. O—O chromatin-bound and ●---● soluble activity from 96-hour hypocotyls; ●—● soluble activity from 48-hour embryos.

Figure 3. Effect of N-ethylmaleimide on DNA polymerase activity O—O chromatin-bound and ●---● soluble activity from 96-hour hypocotyls; ●—● soluble activity from 48-hour embryos.

The 48-hour and 96-hour soluble enzymes exhibited a different response to varying concentrations of N-ethylmaleimide (NEM, Fig. 3). The 48-hour soluble enzyme was inhibited by NEM concentrations as low as 0.25 mM. In contrast, the 96-hour soluble enzyme was stimulated 30% at 0.5 and 1.0 mM concentrations. The chromatin enzyme was relatively unaffected by NEM up to a concentration of 1.0 mM. All three enzymes were considerably inhibited (85%) with higher NEM concentrations (4.0 mM).

Although all three enzymes fractions efficiently used an activated calf thymus DNA template (Table 1), both soluble enzymes (48- and 96-hour) were more active with a poly dA·dT template. When a synthetic RNA template was used, the 96-hour soluble and chromatin fractions exhibit low levels of activity whereas the 48-hour soluble enzyme showed a relatively high rate of ^{3}H-TMP incorporation (86% of control).

Assays to distinguish the types of DNA polymerases (Table 2) indicated that for all three fractions most activity appeared as α-polymerase. In the 96-hour fractions α-activity accounted for 88% of the activity in the soluble fraction but only 66% of the activity in the chromatin fraction. Of the total polymerase activity present in the 96-hour tissue, 74% was observed as

Table 1. Activity of soybean DNA polymerase fractions on various template-primer systems

Template/primer*	Soluble 48-Hour % of Control Activity	Soluble 96-Hour % of Control Activity	Chromatin 96-Hour % of Control Activity
Activated DNA	100	100	100
Poly(dA) ∘ dT_{12-18}	139	139	68
Poly(rA) ∘ dT_{12-18}	86	45	31

*Template concentrations used in these experiments were 10 µg/assay.

α-activity and 26% as β-activity. No DNA polymerase-γ activity was observed in any of the preparations. The presence of β-activity in the soluble fraction may indicate some nuclear disruption in the isolation procedure.

Discussion: The onset of soybean seed germination was characterized by a rapid increase in the amount of DNA polymerase activity, especially in the soluble fraction, which was detectable at six hours. This appeared similar to other systems where an active polyribosome system was present in wheat embryos 30 min after the onset of germination (16), but where DNA synthesis was not observed before 12-15 hours (17).

Table 2. Activity of various soybean fractions in specific DNA polymerase assays

Assays*	Soluble 48-Hour	Soluble 96-Hour	Nuclear 96-Hour
α - cpm	13,898	16,480	22,230
% of total activity	96	88	66
β - cpm	516	2,205	11,485
% of total activity	4	12	34

*Specific assays are as described in Materials and Methods.

Table 3. Comparison of soluble 96-hour DNA polymerase activity from soybean with DNA polymerase-α from other sources

Characteristic	Soybean	Pea[7]	Crown Gall Tumor[18]	Human KB Cells[19]
pH optimum	7.5-8.0	7.9	7.5	7.6
Optimum for:				
Mg^{2+} (mM)	10	15	6	10
Mn^{2+} (mM)	inhibited	1.0	0.5	0.2
Inhibition by:				
N-ethylmaleimide (mM)	>2.0	0.25	1.0	inhibited
KCl (mM)	>200	-	-	>200
Stimulation	50-100	25	50	50-100

A comparison of the 48-hour and 96-hour soluble enzymes from soybean indicated several characteristics such as in metal cation requirements and sensitivity to N-ethylmaleimide and KCl, which may reflect alterations in enzyme regulation during development or the existence of multiple forms of soluble polymerase as in wheat embryos (4) and sugar beet (8). These changes may also reflect qualitative differences in the types or regulation of polymerases which were obtained from the entire embryonic axis (48-hour fraction) compared to the hook region of the hypocotyl (96-hour fraction).

Table 4. Comparison of chromatin-bound DNA polymerase activity from soybean with DNA polymerase-β from other sources

Characteristic	Soybean	Pea[7]	Sugar Beet[8]	Novikoff Hepatoma[20]
pH optimum	8.0	7.3	8.0	8.4
Optimum for:				
Mg^{2+} (mM)	10	15	10	5-10
Mn^{2+} (mM)	0.25	1.0	-	1.0
N-ethylmaleimide inhib. (mM)	1.0	2.0	0.25	5.0
KCl stimulation (mM)	50-100	50-175	50-100	50-100

A comparison of the 96-hour soluble and chromatin-bound enzymes from soybean with DNA polymerase α and β, respectively, from other tissues indicates that the soluble enzyme resembles DNA polymerase-α (Table 3) and the chromatin-bound enzyme is similar to DNA polymerase-β (Table 4). Plant DNA polymerase-β may be more sensitive to the action of N-ethylmaleimide than polymerases from other sources and, therefore, should not be the sole basis for distinction of the type of polymerase present. The presence of 42% of the α-activity in the soluble fraction may be a result of nuclear breakage, leakage from the nuclei (21) or newly synthesized enzyme present in the cytoplasm of this rapidly growing tissue.

Data presented in this study indicate the presence of active DNA polymerases as early as six hours following the onset of seed germination. DNA polymerase-α increases rapidly during germination as seen in other systems undergoing rapid cell division. In addition, it is possible that the activity of DNA polymerases during germination may be regulated by altered sensitivity to cations.

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