

A Rapid Technique for the Estimation of Polynucleotide Adenylyltransferase and Ribonucleic Acid Polymerase in Plant Tissues¹

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ABSTRACT

Nucleic acid-dependent polynucleotide adenylyltransferase (EC 2.7.7.19) and ribonucleic acid polymerase (EC 2.7.7.6) have been partially purified from maize tissues (*Zea mays* L.) utilizing ammonium sulfate precipitation and batch diethylaminoethylcellulose chromatography. The technique is applicable to the simultaneous processing of up to eight samples of plant tissue and affords a rapid and reproducible means of assaying these two enzymes from small quantities of kernels or seedlings. The kinetic characteristics of the partially purified enzymes resemble those from more extensively purified preparations.

steps are time-consuming, limited to analysis of a single tissue sample, and often result in considerable losses of total activity. We have developed a rapid, small scale, three-step purification procedure with which we can cope with many tissue samples at once. Nucleic acid-dependent exotransferase and RNA polymerase activities can be correlated with nucleic acid metabolism in developing tissues.

MATERIALS AND METHODS

Plant Materials. Waxy maize (*Zea mays* L., WF9 × Bear 38) was grown to maturity in a greenhouse and hand-pollinated (7). Ears of corn were collected at intervals after pollination, the kernels stripped, frozen in liquid nitrogen, and stored at -60 C. *Zea mays* L. (WF 9 × Bear 38, waxy, MS) was germinated for 4 days, and the roots and shoots were isolated as described previously (18).

Buffers. Buffer A contained 0.1 M tris·HCl, pH 8 at 25 C, 5% (v/v) glycerol, 0.1 mM MgCl₂, 20 mM 2-mercaptoethanol and 0.7 M (NH₄)₂SO₄. Buffer B contained 2.5 mM tris·HCl (pH 8 at 25 C), 5% (v/v) glycerol and 1 mM 2-mercaptoethanol. Buffer C was buffer B with 10 mM (NH₄)₂SO₄.

Gel Filtration. Salt and other low mol wt substances were removed from aliquots of the indicated fractions by passage through Sephadex G-25 (4 × 0.9 cm), equilibrated, and eluted with buffer C, at a flow rate of 5 ml/hr. Prior calibration with blue dextran (Pharmacia) and sodium benzoate established that the high mol wt components eluted after 1.1 ml void volume and in 0.9-ml excluded volume. Desalted fractions were collected and stored in liquid nitrogen with no loss in activity.

Enzyme Purification. Frozen tissue (10-12 g of kernels or roots and shoots) was homogenized with 18 to 22 ml of buffer A at 0 C in a VIRTIS 45 homogenizer at a speed setting of 50 for 60 sec and then at a speed setting of 100 until complete disintegration of large debris was observed. The second homogenization was typically 10 sec for seedlings and 0 to 8-day-old kernels and up to 60 sec for hard tissues, such as 40-day-old kernels. All further steps were at 0 to 4 C.

The homogenate was filtered through gauze and the debris washed with 4 ml of buffer A; the combined filtrates were centrifuged at 40,000g for 20 min, and the supernatant fluid was filtered through Miracloth (fraction I). The high (NH₄)₂SO₄ concentration in buffer A precluded a direct assay in fraction I. Therefore, an aliquot (0.5 ml) was desalted as described above prior to assay.

Saturated (NH₄)₂SO₄ (0.8 volumes) was added with stirring to fraction I. The precipitate was collected after 30 min by centrifugation at 20,000g for 10 min and dissolved in a minimal volume (2-4 ml) of buffer B (fraction II). Fraction II was between 0.4 and 0.8 M (NH₄)₂SO₄, determined by conductivity measurement. An aliquot (0.5 ml) of fraction II was desalted prior to assay.

Poly(A)² sequences have been found in eukaryotic mRNA and HnRNA (2, 4, 13), in viral RNA (5, 6, 10), and in plant RNA (8, 9, 15, 28). Polynucleotide adenylyltransferase (exotransferase, EC 2.7.7.19) which covalently links AMP moieties from ATP to the 3'-hydroxyl terminus of RNA primers has also been found in many of these tissues (3, 11, 12, 21, 27, 30). The enzyme isolated from maize seedlings (29) will synthesize poly(A) chains on several nucleic acid primers, including tRNA (22), DNA (16, 19), and histone mRNA (26). The use of mRNA as a primer for poly(A) synthesis by the maize exotransferase suggests that this enzyme is also active *in vivo*. The relationship between RNA and poly(A) synthesis and the role of the poly(A) segment are obscure. Changes in eukaryotic RNA polymerases (24) and exotransferase activities correlated with physiologic responses observed during naturally occurring (differentiation, development) or induced (virus infection, illumination) alterations in the utilization of genetic information may provide insight into the role of poly(A).

Potentially, differentiating plant tissues offer an ideal material for this approach. However, the ubiquitous presence of hydrolytic activities, RNases and proteases in particular, the inhibition by the endogenous nucleotides, the presence of unrecognized inhibitors and activators, and the lack of primer and template specificity render an *in vitro* assay of crude homogenates difficult to interpret. Preparative enzyme purifications entailing several

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² Abbreviations: poly(A): polyadenylic acid; HnRNA: heterogeneous nuclear ribonucleic acid.

Fraction II (1 ml) was diluted with sufficient buffer B to reduce the $(\text{NH}_4)_2\text{SO}_4$ concentration to 0.15 M. DEAE-cellulose (1.9-ml packed volume prepared according to Peterson and Sober (23) and equilibrated with buffer B) was added and equilibrated with fraction II for 10 min. The suspension was transferred to the barrel of a 5-ml syringe containing glass wool in the tip. The syringe was then placed in a 50-ml centrifuge tube and the flow-through solution collected by centrifugation at 1,000g for 5 min (fraction III). An aliquot (0.5 ml) of fraction III was desalted as described.

Protein was determined on trichloroacetic acid-precipitated fractions by the method of Lowry *et al.* (14) with BSA as a standard.

Enzyme Assays. Polynucleotide adenyltransferase was assayed in standard reaction mixtures (0.05 ml) containing 70 mM tris-HCl, pH 8.8 at 25 C, 25 mM 2-mercaptoethanol, 1 mM $[8\text{-}^{14}\text{C}]\text{-ATP}$ (2 to 4 Ci/mole), 1 mM MnCl_2 , 1 mg/ml BSA, 10 mM $(\text{NH}_4)_2\text{SO}_4$, 0.22 A_{260} /ml tRNA and 0.1 to 0.4 mg/ml enzyme protein. One unit of activity is defined as 1 pmole AMP incorporated in 1 min at 30 C. RNA polymerase was assayed in standard reaction mixtures (0.1 ml) containing 100 mM tris-HCl, pH 8 at 25 C, 10 mM 2-mercaptoethanol, 1 mM $[8\text{-}^{14}\text{C}]\text{UTP}$ (6 to 8 Ci/mole), 2.4 mM ATP, CTP, and GTP each, 1 mg/ml denatured calf thymus DNA, 5 mM MnCl_2 , 80 mM $(\text{NH}_4)_2\text{SO}_4$ and 1 to 4 mg/ml enzyme protein. One unit of activity is defined as 1 pmole UMP incorporated in 1 min at 30 C. Incorporation into acid-insoluble material was measured on filter paper discs (20) as modified previously (18).

RESULTS

Reproducibility of Purification Procedure. Triplicate preparations from kernels harvested 33 days after pollination were compared along with the purification of exotransferase from 4-day-old roots and shoots. Highly reproducible specific activities and recoveries were obtained at each stage of the replicate kernel purification (Table I). The exotransferase activity was estimated after gel filtration of an aliquot of fraction I and was arbitrarily designated as 100%. Some loss in activity was seen after selective precipitation of fraction I with $(\text{NH}_4)_2\text{SO}_4$ (fraction II), but fraction I included several ATP-incorporating activities other than exotransferase. The yield of fraction II exotransferase activity was reproducible within the experiment. Similar yields were obtained from the same tissue on repeated analyses. Little loss of activity occurred with DEAE-cellulose purification of kernel fraction II (fraction III). An apparent loss of the seedling activity occurred at this stage if assayed prior to removal of low mol wt components. Although not seen with activity from older kernels, the stimulation in specific activity upon desalting

Table I Purification of Kernel and Seedling Exotransferase

Preparations from kernels collected 33 days after pollination (each entry represents the mean and standard deviation for 3 replicates) and a preparation from 4-day-old seedlings were purified and assayed as described under "Materials and Methods."

Tissue	Frac-tion	Total Protein mg	Activity		Yield %
			Specific units/mg	Total units	
Kernel	I	68.5 ± 4.1	92.4 ± 2.6	6449 ± 439	100
Kernel	II	36.9 ± 1.2	101.4 ± 1.9	3734 ± 177	58
Kernel	III	32 ± 2.8	130.7 ± 2.4	3642 ± 305	56
Seedling	I	49.6	191.1	9479	100
Seedling	II	23	278.9	6714	68
Seedling	III	14.4	410	5904	62

was also observed with kernels isolated soon after pollination; desalting of fraction III is required for the accurate assessment of exotransferase activity in developing tissues.

Exotransferase Characterization. Exotransferase isolated by the procedure described here was stimulated significantly by added primer (Table II). Major differences in the extent of primer dependence were detected in pre-DEAE-cellulose fractions from kernels as compared with those from roots and shoots.

The time course of AMP incorporation with the seedling enzyme showed a lag in the initial stages of the reaction and then rapid AMP incorporation continued throughout the 4-hr incubation period (Fig. 1, panel A); typical of the maize exotransferase (29). Activity from kernels exhibited no consistent lag initially and continued at a slower rate for 4 hr. Note the reproducibility among three preparations of kernel fraction III. Maize seedling exotransferase has the ability to utilize deoxyoligomer primer, as well as RNA primers (19). The kernel fraction III also exhibited this broad primer specificity (Fig. 1, panel B), although AMP addition to the deoxyoligomer was about one-third that seen with the tRNA. The kinetics of deoxyoligomer-primed AMP

Table II. Primer Dependence of AMP Incorporation

Fractions were isolated from kernels collected 30 days after pollination and from 4-day-old seedlings and assayed in standard reaction mixtures with or without tRNA. Fractions I and III were desalted prior to assay; fraction II was not. Per cent primer dependence was calculated as the activity (with - without/with) 100.

Fraction	Specific Activity					
	Kernel			Seedling		
	Without	With	Depend- ence	Without	With	Depend- ence
	units/mg		%	units/mg		%
I	6.7	62.2	89	86.7	191.1	55
II	5.6	96.7	94	140.0	278.9	50
III	8.9	134.4	93	54.4	410.0	87

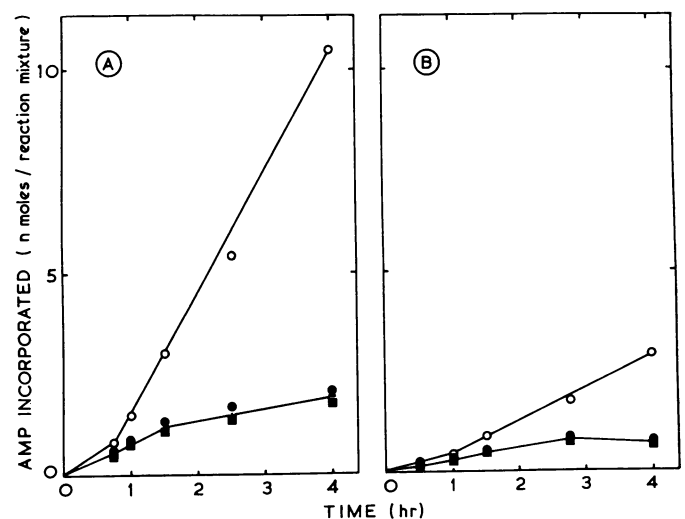


FIG. 1. Time course of AMP incorporation. A 4-fold standard reaction mixture containing 109 μg (●—●), 104 μg (▲—▲) or 85 μg (■—■) of fraction III from kernels collected 33 days after pollination or 73 μg of fraction III from 4-day-old seedlings (○—○) and either 0.22 A_{260} /ml tRNA (A) or 0.22 A_{260} /ml deoxyoligomer (B) was incubated at 30 C. Aliquots were removed at the times indicated and the amount of acid-insoluble radioactivity determined.

incorporation are similar to that with tRNA with either seedling or kernel fraction III proteins, although no further incorporation with the kernel protein was seen after 3 hr.

The specific activity of the seedling fraction III was independent of protein concentration between 0.4 and 1.2 mg/ml (Fig. 2, panel A). In contrast, the specific activity of the kernel fraction III decreased with increasing protein concentration between 0.2 and 1.2 mg/ml. At lower protein concentrations (0.1–0.2 mg/ml), the kernel fraction III specific activity was constant. The effect of increasing the concentration of protein on the specific activity of the DNA-primed reaction mimicked that seen with the tRNA-primed reaction (compare Fig. 2, panel A with B).

Mixing Experiments. The marked difference in specific activity between the kernel and seedling preparations and the decrease in specific activity with increase in protein concentration of the kernel preparation suggested the presence of an inhibitory component in the kernel preparation. However, analysis of mixtures of seedling and kernel enzymes, in varying proportions, resulted in additive incorporation of AMP, measured after 30 min incubation (end of lag) or after the standard 90 min incubation (Fig. 3). Thus, the observed difference in specific activity between kernel

and seedling fraction III proteins cannot be ascribed to inhibitors or activators detectable by mixing experiments.

RNA Polymerase. Quadruplicate preparations from kernels collected 30 days after pollination were compared with the purification of RNA polymerase from 4-day-old seedlings (Table III). Highly reproducible activities were obtained in fractions I and II, although one-half the α -amanitin sensitive incorporation was lost upon $(\text{NH}_4)_2\text{SO}_4$ precipitation. UMP incorporation by kernel and seedling fraction II protein was directly proportional to the amount of protein added to a standard reaction (Fig. 4), but the incorporation was not linear with time during the 20-min incubation. RNA polymerase activity in fraction II was identi-

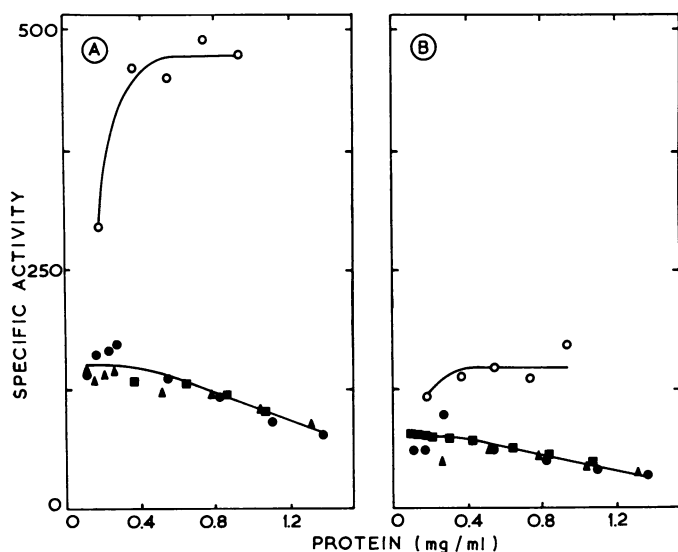


FIG. 2. AMP incorporation as a function of protein concentration. Standard reaction mixtures containing the indicated amount of fraction III from triplicate preparations from kernels collected 33 days after pollination (closed symbols) or from 4-day-old seedlings (open symbols) and either 0.22 A_{260} /ml tRNA (A) or 0.22 A_{260} /ml deoxyoligomer (B) were incubated at 30 C for 90 min and the acid-insoluble radioactivity determined.

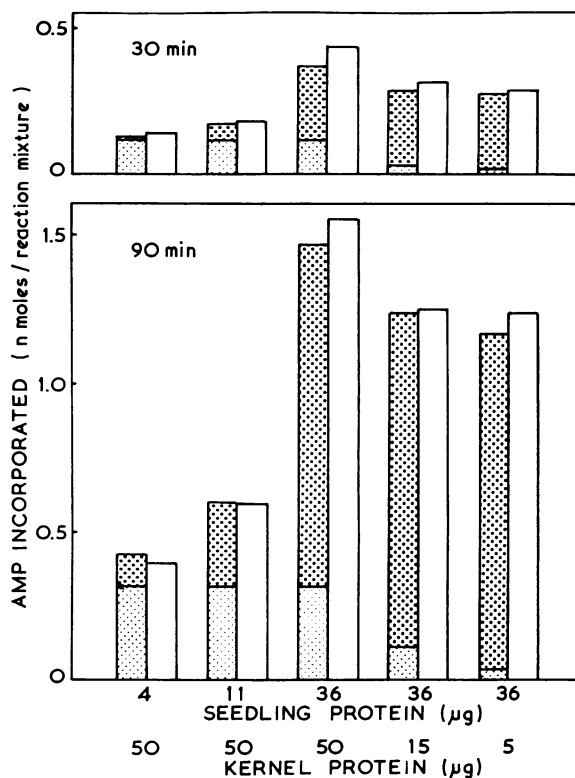


FIG. 3. Activity upon mixing kernel and seedling fraction III proteins. Two-fold standard reactions containing fraction III from kernels collected 33 days after pollination and from 4-day-old seedlings were incubated at 30 C and the amount of acid-insoluble material was determined in aliquots taken 30 and 90 min after enzyme addition (line histogram). Expected AMP incorporation by kernel fraction III (hatched histogram) or seedling fraction III (stippled histogram) was calculated from AMP incorporated by incubation of each preparation alone.

Table III. Purification of Kernel and Seedling RNA Polymerase

Preparations from kernels collected 30 days after pollination (each entry represents the mean and standard deviation for four replicates) and a preparation from 4-day-old seedlings were purified and assayed in standard reaction mixtures with or without α -amanitin (0.4 $\mu\text{g}/\text{ml}$). Yield was calculated from the total, α -amanitin-sensitive, UMP incorporation assuming fraction I as 100%.

Tissue	Fraction	Total Protein mg	Specific Activity units/mg		Total units		Yield %
			Without	With	Without	With	
Kernel	I	92.6 \pm 2.7	11.85 \pm 0.07	2.52 \pm 0.05	1097 \pm 48	233 \pm 4	100
Kernel	II	54.6 \pm 1.5	10.66 \pm 0.63	2.14 \pm 0.06	556 \pm 37	117 \pm 4	51
Seedling	I	81	66	23.5	5346	1904	100
Seedling	II	55.9	36.4	10.3	2035	585	38

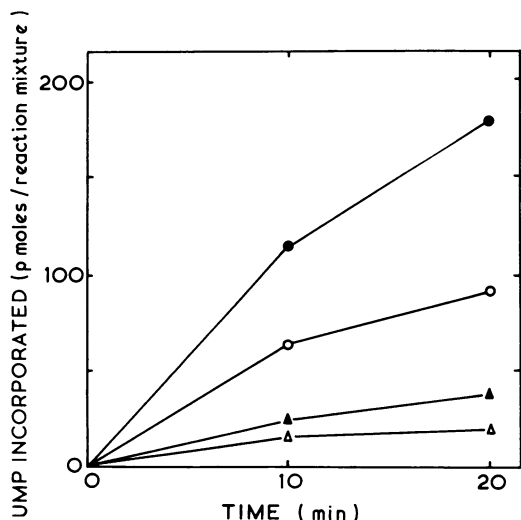


FIG. 4. Time course of UMP incorporation by seedling and kernel RNA polymerase. Standard reaction mixtures containing 1.8 mg/ml (○—○) or 3.6 mg/ml (●—●) of seedling fraction II or 1.1 mg/ml (△—△) or 2.2 mg/ml (▲—▲) of kernel fraction II were assayed for RNA polymerase as described under "Materials and Methods." Activity is expressed as α -amanitin sensitive UMP incorporation.

Table IV. Characteristics of RNA Polymerase in Kernel and Seedling Fraction II

A complete system contained 380 μ g of fraction II proteins prepared and assayed as described in Table III; α -amanitin (0.4 μ g/ml) or actinomycin D (10 μ g/ml) were added as indicated. Values are given as the mean of four preparations with the standard deviation where indicated.

System	Kernel		Seedling	
	Specific activity	Inhibition	Specific activity	Inhibition
	units/mg	%	units/mg	%
Complete	10.66 \pm 0.63	0	36.4	0
Plus α -amanitin	2.14 \pm 0.06	80	10.3	72
Plus actinomycin D	1.75	84	8.5	77
Minus NTPs	2.05	81	28.7	21
Minus DNA	5.65	47	9.8	73

fied as the UMP incorporation sensitive to α -amanitin or actinomycin D (Table IV). Kernel fraction II RNA polymerase was not completely dependent on added DNA, suggesting the presence of substantial amounts of endogenous DNA. Four-fifths of the UMP incorporation observed with the seedling fraction II was independent of added NTPs, whereas most of the kernel fraction II activity was NTP-dependent.

DISCUSSION

Seedling exotransferase isolated by the purification procedure described in this report had a higher specific activity (410 units/mg) than that isolated previously by a preparative procedure (67–134 units/mg, ref. 29). The speed of isolation with the present method, the inclusion of 0.7 M $(\text{NH}_4)_2\text{SO}_4$ in the homogenization buffer to precipitate polysaccharide, and the use of higher $(\text{NH}_4)_2\text{SO}_4$ concentrations for the precipitation of fraction II proteins account for the higher activity and the reproducibility of exotransferase preparations, thereby enabling the purification procedure to be extended to kernels. The greater primer dependence exhibited by the kernel as compared to the seedling

exotransferase probably reflects higher levels of endogenous nucleic acid in the more active seedling as compared with kernels harvested late in maturation. Removal of endogenous nucleic acids with DEAE-cellulose renders both kernel and seedling equally primer-dependent enabling poly(A) synthesizing activity to be measured independent of the nucleic acid composition of the tissue.

The decrease in specific activity observed with high concentrations of kernel exotransferase and the decrease in the rate of AMP incorporation between 3 and 4 hr incubation suggests a nuclease contaminant. By the use of low concentrations of kernel protein, valid assays of synthetic activity can be obtained. Although mixing experiments failed to demonstrate significant inhibitory activity (owing to the putative nuclease), a direct assay of kernel fraction III using labeled poly(A) as substrate indicated nuclease activity.

It is significant that the kernel enzyme exhibited the same broad primer specificity as the seedling (16) in contradistinction to the strict RNA specificity of the mammalian enzymes (27, 30). Although the occurrence of poly(A) in covalent attachment with DNA is unknown *in vivo* at present, the isolation of such an enzymic activity in developing tissues suggests a physiologic role for it may exist.

Since DNA-dependent, α -amanitin-sensitive RNA polymerase activity can be measured in fraction I or II in addition to exotransferase, a correlation of these two enzymic activities can be obtained by application of this assay to tissues in different physiologic states. The equivalence of α -amanitin-sensitive and actinomycin D-sensitive nucleotide incorporation strongly indicates that only RNA polymerase II activity could be detected in the kernel and seedling preparations.

The lack of DNA dependence of half of the UTP incorporating activity by the kernel fraction II was attributed to endogenous DNA. Taken together with the lack of primer dependence for seedling exotransferase activity, the data suggest that the kernels are rich in DNA templates and the seedlings are rich in RNA and DNA primers. Since exotransferase uses only single-stranded DNA as primer (16) and since the maize RNA polymerase II will utilize native DNA (25), the endogenous template in the kernel fraction II is probably native DNA. Lack of dependence of NTPs for UMP incorporation is also observed with AMP incorporation. The NTP-independent UMP incorporating activity is not exotransferase in fraction II since this enzyme does not incorporate UMP but may be analogous to the UTP-exotransferase found in rat liver (11). The NTP-independent AMP incorporation may be ascribed to template-dependent poly(A) synthesis by maize RNA polymerase II (1; R. H. Benson and R. J. Mans, manuscript in preparation).

Since we have developed a rapid, three-step, purification procedure for small amounts of many tissues simultaneously, we can now scan nucleic acid transitions in developing tissues. By the judicious use of specific inhibitors of RNA polymerase and exotransferase (17) in these assays, we should elucidate the relationship between RNA and poly(A) synthesis.

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