RNA POLYMERASE III FROM WHEAT EMBRYOS

Purification by affinity and hydrophobic chromatographies

Characterization and molecular properties

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1. Introduction

It is now clearly established that higher plant cells contain diverse classes of DNA-dependent RNA polymerases of nuclear origin. Each class is thought to function similarly to its counterpart in other eukaryotes [1].

Class B (or II) enzymes, which are generally the major components, have been purified from several plant sources and their subunit structure has been determined [2–5]. Recently, the purification and the determination of the subunit structure of a class A (or I) RNA polymerase from soybean were achieved [6]. Class C (or III) RNA polymerase has not been characterized in most of the plant tissues so far investigated. However, we have previously reported that lentil roots [7] and wheat embryos [8,9] contain three types of DNA-dependent RNA polymerase activities: Ia and Ib which are insensitive to α-amanitin, II which is inhibited by low concentrations of this drug, and a third one which is 50% inhibited by 10 μg/ml of α-aminitin and which could be tentatively identified with RNA polymerase III as defined for animals and lower eukaryotes [10]. Nevertheless, to confirm the latter point it was necessary to compare the subunit structure of this enzyme with those of RNA polymerases I and II, for it has been reported that wheat germ would contain only enzymes I and II [11].

In this paper, we describe a method of purification of RNA polymerase III from plant material based on affinity and hydrophobic chromatographies which allowed the characterization of the structure of this enzyme.

2. Materials and methods

Raw, freshly milled wheat germ (provided by Grands Moulins d'Arenc-Marseille) was purified as described by Johnston and Stern [12] to obtain pure and viable wheat embryos.

Heparin (Sigma 165 units/mg) was covalently bound to Sepharose 4B (Pharmacia Fine Chemicals, Uppsala, Sweden) as previously described [12,14]. The agarose gel (100 ml) was activated by cyanogen bromide, then mixed with heparin (500 mg dry wt) dissolved in a solution (150 ml) containing 100 mM carbonate buffer, pH 8.5, 0.5 M NaCl. The mixture was gently stirred for 2 h at 20°C, then filtered on a Büchner funnel and washed with 250 ml of the same buffer. The gel was suspended in 250 ml 100 mM acetate buffer, pH 4, 1 M NaCl and 250 ml 100 mM carbonate buffer, pH 8.5, 1 M NaCl. Heparin–Sepharose was then equilibrated with standard buffer containing 50 mM Tris–HCl, pH 7.8, 5 mM $MgCl_2$, 0.1 mM EDTA, 10 mM thioglycerol, 25 mM 2-mercaptoethanol, 25% (v/v) glycerol plus 0.1% (w/v) Triton X-100.

The heparin content of the gel (380 units/ml) was

evaluated by colorimetric analysis of supernatant of reaction mixtures by the method of Jaques and Bell [15]. δ-Aminobutyl–Sepharose was prepared according to Shaltiel and Er-El [16]. RNA polymerase activity was assayed with the mixture previously described [7] in a final vol. 100 μl, denatured calf thymus DNA was used as template (15 μg). One unit of activity is defined as 1 nmol UMP incorporated into RNA in 30 min at 35°C.

Protein concentration was determined either after trichloroacetic acid precipitation, as previously described [7] or by densitometry of the bands stained with Coomassie Brilliant Blue after polyacrylamide gel electrophoresis. Salt concentrations were measured by conductimetry using a Metrohm Herisau E 382 conductimeter following a 1000-fold dilution of the samples with water. Gel electrophoresis of the protein samples was conducted under denaturing conditions in 0.3 × 8.5 × 10 cm polyacrylamide slabs in a Desaga slab gel apparatus using the buffer system of Laemmli [17]. The resolving gel contained a linear 6–15% acrylamide gradient. In some experiments, to determine the molecular weight of the subunits, the electrophoresis was carried out in discontinuous gels (8.5% acrylamide in the upper half of the gel and 13% below). Molecular weight standard used were *E. coli* RNA polymerase subunits β′ (165 000) and β (155 000), β-galactosidase (130 000), phosphorylase A (94 000) bovine serum albumin (68 000), *E. coli* RNA polymerase subunit α (39 000), trypsin inhibitor (21 500), cytochrome *c* (12 500). After electrophoresis, the gels were stained and destained according to Sklar and Roeder [18]. The stained bands were scanned with a Gilford 2400 S spectrophotometer with a linear transport device.

3. Results

3.1. *Initial purification*

All operations are carried out at 4°C. After grinding the purified wheat embryos (250 g) with a Waring Blendor in a buffer (1500 ml) containing 100 mM Tris–HCl, pH 7.8, 15 mM magnesium acetate, 1 mM $MnCl_2$, 25 mM 2-mercaptoethanol and 10 mM thioglycerol, the solution is filtered on plankton netting with a 60 μm mesh. The suspension is adjusted to 0.5 M ammonium sulfate and gently stirred for 3 h, then centrifuged at 47 000 × *g* for 30 min. The 0.5 M supernatant is fractionated with ammonium sulfate at 40% saturation. The 40% precipitate is resuspended in the standard buffer (150 ml) containing 0.1% Triton X-100. The suspension is dialyzed overnight against 4 liters of standard

Table 1
Purification of RNA polymerase III from wheat embryos

Purification step	Protein (mg)	Activity (unit)	Specific activity (unit/mg)	Recovery (%)
I. Solubilisation and initial purification (Total activity, i.e., class I + II + III)				
Homogenate	45 000	160	0.0036	–
40% Ammonium sulfate precipitate[a]	12 250	230	0.019	–
1st Heparin–Sepharose	1000	1270	1.27	100
II. Separation and purification (Class III activity)				
DEAE–Sephadex	12.3	57.4	4.67	4.5
2nd Heparin–Sepharose	4.2	56.1	13.3	4.4
δ-Aminobutyl–Sepharose	0.45	55.3	123	4.3

[a]After suspension, dialysis against standard buffer and a 30 min centrifugation at 47 000 × *g*

RNA polymerase activity and protein concentration were determined as indicated in the technical section. Only 90% of the activity which was recovered at each stage was actually employed for the subsequent purification step. Data were calculated assuming that no activity had been discarded in each of the various steps. The data reported here were taken from one representative experiment starting with 250 g wheat embryos

buffer containing 0.1% (w/v) Triton X-100, then centrifuged for 30 min at 47 000 × *g*. Supernatant (250 ml) is applied to a column of heparin–Sepharose. The RNA polymerase activity is eluted at 0.45 M ammonium sulfate [8]. Heparin–Sepharose chromatography removes about 90% of the contaminating proteins in the ammonium sulfate precipitate (table 1) and gives an apparent purification of 60-fold. The strong increase in total activity is probably due to the removal of inhibitors. This emphasizes the difficulty of estimating the actual level of RNA polymerase in the whole embryos extract.

The fractions containing the major part of the total RNA polymerase activity are pooled, dialyzed against standard buffer, then loaded onto a DEAE–Sephadex A 25 column as described elsewhere [8]. The elution is performed with a linear ammonium sulfate gradient (0–0.6 M) in standard buffer. Four fractions Ia, Ib, II and III are eluted from this column. The yield of this chromatography is about 70% for the total RNA polymerase activity; RNA polymerase III represents only 5% of the activity recovered (table 1).

3.2. *δ-Aminobutyl–Sepharose chromatography*

The active fractions of RNA polymerase III (~ 160 ml) purified by DEAE–Sephadex chromatography are concentrated by affinity chromatography onto a small column of heparin–Sepharose (0.7 × 4 cm). The activity is eluted in about 12 ml with standard buffer containing 0.45 M ammonium sulfate. The yield of this concentration step is about 95%. A 3-fold purification is obtained (table 1). After a 3 h dialysis against the standard buffer, the enzyme is applied to a δ-aminobutyl–Sepharose column. The RNA polymerase activity is eluted in one peak at 0.12 M ammonium sulfate (fig.1).

This step gives an additional 9-fold purification with a yield of 94%. Specific activities measured across the elution peak average 125 units/mg. Assuming that the relative amount of enzyme III in the crude extract (45 000 mg protein/250 g embryos) is the same as that estimated from the DEAE–Sephadex flow sheet and taking into account the loss of activity (about 30%) in this step one can estimate the specific activity of RNA polymerase III in the crude extract to be 0.002 units/mg. Thus RNA polymerase III from wheat embryos is purified approximately 60 000-fold relative to the crude extract.

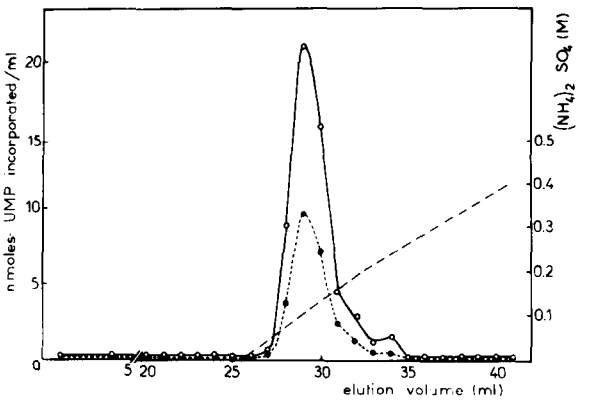

Fig.1. δ-Aminobutyl–Sepharose chromatography of wheat embryo RNA polymerase III. After concentration on heparin Sepharose, the pooled fractions (12 ml) containing 3.8 mg protein, 54 units enzyme were dialyzed against standard buffer in which 2-mercaptoethanol was replaced by 5 mM dithiothreitol then loaded onto a 0.7 × 13 cm column of δ-aminobutyl–Sepharose. The column was washed with 10 ml same buffer, then eluted with a linear ammonium sulfate gradient (0–0.5 M in 20 ml) in the same buffer. Fractions, 1 ml, were collected. Each fraction was assayed either in absence (o——o) or in the presence (•- - - -•) of 10 μg/ml of α-amanitin. Ammonium sulfate concentration (— —).

δ-Aminobutyl–Sepharose fractions were assayed either in absence or in the presence of 10 μg/ml of α-amanitin (fig.1). The partial inhibition brought about by the toxin (55%) is the same for each active fraction. That this enzyme is free of class I and class II activities can be verified by the analysis in polyacrylamide gel electrophoresis under denaturing conditions (fig.2). It appears that the polypeptides detected are nearly the same for the three fractions containing the highest level of enzyme activity. A densitogram of a polyacrylamide gel electrophoresis is presented in fig.3.

One can observe two large polypeptides with molecular weight estimated at 155 000 (III a) and 132 000 (III b), respectively and 10 additional bands estimated at 91 000 (III c), 70 000 (III d), 66 000 (III e) 53 000 (III f), 37 000 (III g), 33 000 (III h), 31 000 (III i_1), 28 000 (III i_2), 26 000 (III i_3), 16 000 (III i_4), Components III d and III e could be contaminants, for their stoichiometry is variable along the purification process. Components III a, III b, III c and III g are approximately in equimolar amount.

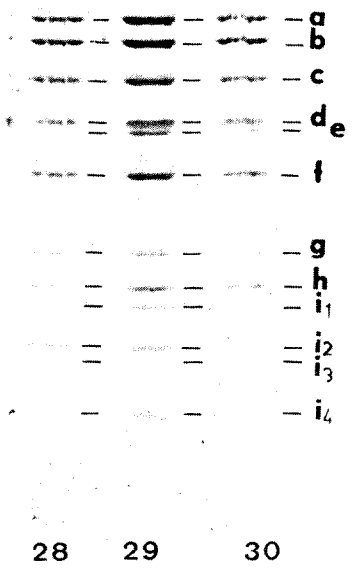

Fig.2. Subunit profile of RNA polymerase III of wheat germ. Aliquots, 30 μl, of the three peak activity fractions (No. 28, 29, 30) of the δ-aminobutyl–Sepharose chromatography corresponding to 0.25, 0.55 and 0.45 units enzyme, respectively, were subjected to electrophoresis on a polyacrylamide gel slab in the presence of sodium dodecyl sulfate. The resolving gel was a linear polyacrylamide gradient 6–15%, 6.5 cm in length, the stacking gel, 1.5 cm in length, contained 3.5% polyacrylamide. Electrophoresis was carried out at 40 mA (constant current) for 2 h. The gel slab was then fixed and stained [18].

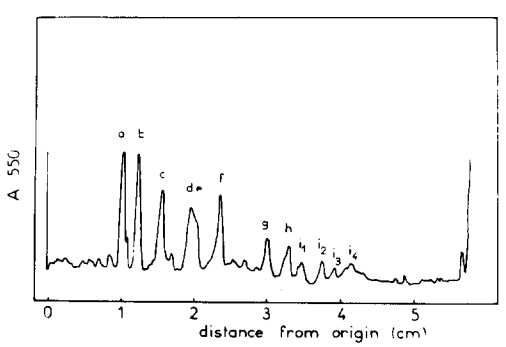

Fig.3. Polyacrylamide gel scanning of RNA polymerase III from wheat embryos. Purified RNA polymerase III, 0.65 units, were electrophoresed on a gel slab as described in the legend to fig.2. The scanning was performed at 550 nm as described in Materials and methods.

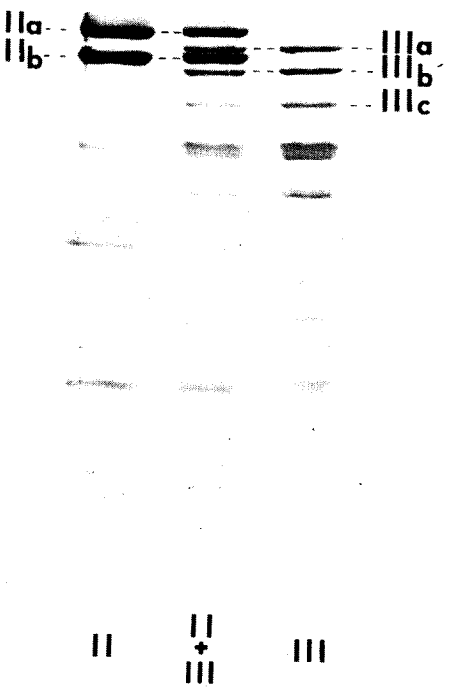

Fig.4. Polyacrylamide gel electrophoresis under denaturing conditions of RNA polymerases II and III of wheat germ. Left, RNA polymerase II (7 μg); center, mixture of RNA polymerase II (3.5 μg) plus RNA polymerase III (5 μg); right, RNA polymerase III (10 μg). These enzymes were electrophoresed as described in the legend to fig.2.

4. Discussion

The use of heparin–Sepharose presents advantages in the purification of RNA polymerases from plant sources. First it yields an important purification at an early stage, then it offers the possibility to concentrate almost without losses the minor component, RNA polymerase III, after the DEAE–Sephadex step. A subsequent chromatography on δ-aminobutyl–Sepharose [19] allows the prepration of this enzyme with a satisfactory yield.

This report provides a structural basis for determining whether the third type of wheat embryos RNA polymerase, which remains sensitive to high concentrations of α-amanitin, actually belongs to the class C enzymes described in other material, and is distinct

from the better studied classes A and B plant RNA polymerases.

The direct comparison of the molecular weight of enzymes III and II from wheat embryos (fig.4) clearly shows that they possess quite different large subunits (III a 155 000, III b 132 000, II a 175 000, II b 145 000). The difference between the forms I and III is more pronounced for enzyme I possesses two large subunits Ia and Ib with mol. wts 195 000 and 125 000, respectively (result not shown).

Although at the present stage of purification it is not possible to decide which of the small 'subunits' could be contaminants, it seems that component III e belongs to the class of 'intermediary components' (molecular weight ranging from 70 000–90 000) which could be a characteristic of the class C RNA polymerases isolated from animals and lower eukaryotes [18,20–23].

Our results demonstrate that the structure of the RNA polymerase III from wheat embryos is different from that of enzyme I and II and exhibits a resemblance to RNA polymerases III from other origin. The availability of purified RNA polymerase III from plants would allow detailed studies of the properties of this enzyme. In particular, we would like to see whether it can use intact double stranded DNA as template, as has been found with RNA polymerase C from animal sources [24,25].

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