Purification of DNA-Dependent RNA Polymerases from Methanogens

The RNA polymerases of archaea are multisubunit enzymes consisting of 7 to 12 different polypeptides (1). Two procedures are described for the purification of RNA polymerases from methanogens to near homogeneity . These enzymes were purified at room temperature in an anaerobic chamber (2). These procedures were originally developed to purify the RNA polymerases from *Methanococcus* (3) and *Methanobacterium* (4), but can be applied to the isolation of the RNA polymerases from most methanogens and their relatives (euryarchaeota) (5). The advantages and limitations of these procedures are discussed.

BASIC PROTOCOL

As an initial step the nucleic acids are separated from the enzyme. This is achieved by hydrophobic interaction chromatography (3) or treatment of a soluble extract with PEG 6000 (4, 6).

Phenyl-Sepharose method

Since nucleic acids do not bind to phenyl-Sepharose (7) whereas the RNA polymerase is adsorbed at 4 M NaCl, the method described here provides a convenient means to obtain an RNA polymerase fraction devoid of internal template. This method has been applied for the purification of the RNA polymerases of *M. vannielii, M. thermolithotrophicus* and *Methanosarcina* sp. The enzyme of *Methanolobus* was inactivated by hydrophobic interaction chromatography.

Materials

Buffer A
Buffer B
Purification buffer

1. Suspend 40 g of cells in 80 ml buffer A and disrupt by passing through a French pressure cell at 140 MPa.

2. Clear the cell extract by centrifugation at 20,000 rpm for 20 min at 5°C (Beckman JA 20 rotor).

3. Homogenize the pellet in 40 ml buffer A, pass again through a French pressure cell and centrifuge.

4. Pool the supernatants of both centrifugation steps and apply to a 56 ml bed-volume phenyl-Sepharose column (3 x 8 cm) equilibrated with buffer A.

5. After washing the column extensively with buffer A, elute the RNA polymerase with buffer B.

6. Desalt the eluted fraction by dialysis against purification buffer.

7. Further purification can be achieved by DEAE cellulose-, heparin-cellulose and Mono-Q-FPLC-chromatography. The RNA polymerases from most methanogens bind to these materials and can be eluted by linear salt gradients ranging from 0.05-0.8 M KCl.

8. As a final purification step some enzymes are applied to a linear glycerol-sucrose gradient (5-10% glycerol; 10-30% sucrose; in purification buffer containing 0.5 M KCl) and centrifuged in a Beckman SW 41 rotor for 22 h at 20°C.

PEG method

This procedure, which is a modification of the original protocol of Humphries et al. (6) is based on PEG precipitation of nucleic acids at high salt conditions. The RNA polymerase is purified from the supernatant of the PEG precipitation by conventional chromatographic procedures. This procedure is recommended to purify the enymes from *Methanobacterium. thermoautotrophicum, Methanothermus fervidus, Methanolobus and Archaeoglobus fulgidus.*

Materials

Purification buffer
PEG 6000
40% Glycerol

1. Suspend 10 g cells in 20 ml purification buffer containing 2 M KCl.

2. Lyse the cells twice using a French pressure cell as indicated above.

3. Adjust the cleared extract to a final concentration of 6 percent PEG 6000 using a 30 percent stock solution in 2 M KCl. Stir for 1 h at 0°C.

4. Remove the precipitate by centrifugation at 40,000 rpm in a 60 Ti rotor for 30 min.

5. Adjust the supernatant to 40% glycerol.

6. Dialyze the sample against purification buffer.

7. Clear the extract by centrifugation at 20,000 rpm for 10 min in a JA20 rotor.

8. Apply the suspension to a DEAE-cellulose column (10 x 5 cm).

Further purification was effected as indicated in the phenyl-sepharose method.

Activity assay
The RNA polymerases from most euryarchaeota show good activity in a reaction mixture containing 20 mM Tris-HCl, pH 8.0, 10 mM Mg Cl_2, 20 mM KCl, 1 mM ATP, 0.1 mM UTP and 0.07 µCi α-^{32}P-UTP as labelled nucleotide (specific activity 80 Ci-mol).

The total reaction volume was 100 µl, the template DNA used poly-d(A-T) at 0.1 mg/ml. The optimal salt-concentrations and incubation temperature are indicated in the following table:

Species	$MgCl_2$ (mM)	KCl (mM)	T (°C)
M. vannielii	10	20	45
M. thermolithotrophicus	20	50	55
Mb. thermoautotrophicum strain W	10-30	200	60
Mb. thermoautotrophicum strain Marburg	7.5	175	57
Mth. fervidus	25	200	65
Methanosarcina sp.	20	0	50
Methanolobus sp.	20	50	35-50
Archaeoglobus fulgidus	20	100	55

REAGENTS AND SOLUTIONS

Buffer A
10 mM Tris-HCl, pH 7.5
4 M NaCl
1 mM EDTA

Buffer B
10 mM Tris-HCl, pH 7.5
4 M NaCl
1 mM EDTA
1 M NaCl

Purification buffer

50 mM Tris-HCl, pH 7.5
50 mM KCl
10 mM $MgCl_2$
40 percent glycerol v/v

COMMENTARY

Although some purified RNA polymerases of methanogens are inactivated at a low rate under aerobic conditions, the presence of oxygen should be strictly excluded during purification. The enzymes in crude extracts appear to be more sensitive to inactivation by oxygen. The RNA polymerases of most methanogens are inactivated by treatment with polymin P which has been used to purify the RNA polymerase of *E. coli* (8) and members of the crenarchaeota (9). A possible additional purification step is ss-DNA-agarose-chromatography (10) which can be used instead of or before heparin-cellulose-chromatography. The enzyme of *Methanosarcina* cannot be eluted in active form from DNA-cellulose columns. Futhermore, this enzyme and the *Methanolobus* RNA polymerase are inactivated during sucrose-gradient centrifugation probably due to dissociation into subunits. As a single exception known so far, the enzyme of *Methanothermus fervidus* does not bind to DEAE-cellulose. This step was replaced by phosphocellulose chromatography. In the presence of high concentrations of glycerol most enzymes are stable for several weeks at room temperature. The enzymatic activity can be conserved for years when the RNA polymerases are stored in the vapor of liquid nitrogen.

The enzyme of *M. vannielli* purified by the phenyl-Sepharose method has been shown to bind specifically to the promotor region of both protein-encoding and stable RNA genes. The footprint extends from position -30 to + 20 relative to the transcription initiation site (11-13). The purified RNA polymerases from all archaea are unable to initiate transcription accurately at correct sites although the enzymes isolated from *M. thermolithotrophicus* and *Sulfolobus shibatae* show some specific initiation *in vitro* (3, 14).

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