

Partial Purification of RNA-polymerase from *Bacillus stearothermophilus*

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DNA-dependent RNA-polymerase was partially purified from *Bacillus stearothermophilus*, growing well at 60-65°C, and properties of the enzyme were investigated. Under the appropriate reaction conditions containing magnesium, RNA-polymerase of *B. stearothermophilus* showed novel thermal stability and also high affinity for homologous *B. stearothermophilus* DNA as template. However, such the specificity for template was diminished by the addition of manganese to the reaction system.

INTRODUCTION

DNA dependent RNA polymerase (nucleoside triphosphate: RNA nucleotidyl-transferase, EC 2. 7. 7. 6) has been prepared from various bacteria and higher organisms¹⁻⁷⁾. In elucidating the molecular mechanism of the template recognition of this enzyme, it is interesting to study the specific interaction between these two components at a wide range of temperature covering the melting point of the template. For this purpose, an attempt was made to prepare the DNA dependent RNA polymerase from *B. stearothermophilus*, which grows well at 60-65°C. Many enzymes prepared from this organism have been found to be stable over 60°C^{8,9)}. In this communication, the procedure for preparing the RNA polymerase from the thermophilic bacterium and some properties of the partially purified enzyme will be described.

METHODS AND MATERIALS

Preparation of cells: *B. stearothermophilus* (ATCC 12016) was grown with aeration at 55°C in a medium containing Bacto-tryptone 10g, yeast extract 5g, and NaCl 5g per liter, with pH adjusted to 7.0⁹⁾. Cells were harvested during the logarithmic phase, washed twice with the standard buffer (10mM Tris-HCl, pH 7.8 and 10mM MgCl₂) and stored at -20°C.

Preparation of DNA: DNA of *B. stearothermophilus* was prepared according to the method of Marmur¹⁰⁾ followed by phenol treatment. Cells grown to the logarithmic phase of their growth cycle were harvested by centrifugation and washed once with saline-EDTA (0.15M NaCl-0.1M EDTA, pH 8). About 3g of the washed cells were suspended in 30-35ml of saline-EDTA and 10mg of lysozyme were added. The mixture was incubated at 37°C for 30 to 60min. with occasional

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shaking. The lysis was followed by noting increase in viscosity. After the complete lysis sodium lauryl sulphate was added to a concentration of 0.1% and the mixture was briefly treated at 60°C and chilled. The solution was then shaken for 20-30min. with an equal volume of 80% phenol, followed by centrifugation at 1,400rpm for 5min. The aqueous phase separated was treated once more with phenol as above. Nucleic acids were precipitated by layering about two volumes of ethanol on the aqueous phase and mixing it gently with a stirring rod. The precipitate spooled on the rod was transferred to 10-15ml of 0.015M NaCl-0.0015M trisodium citrate, pH 7.0 (dilute saline-citrate) and removed from the stirring rod. The solution was gently shaken until the precipitate was completely dissolved. The saline-citrate concentration of the solution was then increased to 0.15M and 0.015M respectively, by adding a concentrated saline-citrate solution. To this solution was added heat treated pancreatic RNase to a final concentration of 40 μ g/ml. Following incubation for 30min. at 37°C, the reaction was terminated by adding 1/10 volume of 1% SDS. RNase was removed by treating the solution with phenol, from which DNA was precipitated as described above. The drained DNA was dissolved in the dilute saline-citrate. After adjusting the saline-citrate concentration to 0.15M and 0.015M respectively, the DNA solution was stored in ice-cold.

Assay of the enzyme activity: The standard assay procedure of the enzyme activity was as follows: The reaction mixture contained (in μ moles/ml), Tris-HCl, pH 7.8; 25, MgCl₂; 8, KCl; 25, GTP, CTP, and UTP; 0.1 each, ¹⁴C-ATP; 0.1 (1 μ C/ μ mol), DNA and the enzyme fraction. The total volume was 0.5ml. Following incubation, the reaction was stopped by adding 8 volumes of cold 0.1N perchloric acid and washed twice with the same solution. The radioactivity in the acid-insoluble fraction was measured by a low background counter.

Determination of protein and DNA concentrations: Measurement of the protein concentration was by the method of Lowry *et al*⁽²⁾. DNA concentration was determined by the Burton's diphenylamine method. Optical density at the ultraviolet region was measured by a Zeiss PHQII spectrophotometer.

RESULTS AND DISCUSSION

Partial purification of RNA polymerase

Frozen cells (about 5g by wet weight) were ground with 3 volumes of quartz sand. The disrupted cells were suspended in 20ml of the standard buffer and the suspension was centrifuged for 15min. at 12,000xg. The precipitate was washed with 10ml of the same buffer. The combined supernatant was then centrifuged at 105,000xg for 120min. in a Spinco 40 rotor. To the supernatant, containing about 2mg protein per ml, was added protamine sulphate to a final concentration of 0.01% with constant stirring. The resulting fibrous precipitate of nucleic acids was removed by centrifugation at 3,000rpm for 10min. It was aimed to precipitate the enzyme fraction by increasing the concentration of protamine sulphate and to elute it from the precipitate at a high ionic strength. However, this was

unsuccessful because of the considerable loss of the activity. Accordingly, the above supernatant fraction was directly adsorbed onto a DEAE-cellulose column (1.2×10cm) which had been washed with 2mM potassium phosphate buffer, pH 7.4. After washing the column, adsorbed materials were first eluted with 10mM of the same buffer containing 0.3M KCl and then with 10ml of the buffer containing 0.5M KCl. The activity of the RNA polymerase was only found in the 0.5M KCl eluate, whereas the bulk of the supernatant proteins was eluted at the 0.3M KCl concentration. Therefore, the specific activity was increased to 20 to 30 times of the original solution by this column treatment. The eluate was dialysed against 2mM phosphate buffer and stored at ice cold. The enzyme activity became relatively unstable after the above purification procedure: the activity decreased to about half by overnight storage at 4°C.

It was noteworthy that the enzyme activity was eluted from the DEAE-cellulose column at a relatively high salt concentration. The result agrees with the observation by Stout and Mans⁴ who recovered RNA-polymerase of maize seedlings from a DEAE-cellulose column at 0.45 to 0.5M Tris-HCl, pH 7.6.

Effect of pH on the RNA-synthesizing activity

The pH optimum for the RNA-synthesizing activity of the enzyme was examined by using *B. stearothermophilus* DNA as primer and at 55°C. As shown in Fig. 1, the maximum activity was obtained at pH 7.5 under the standard reaction conditions (see the method section). At pH 6.6 or 9.1, the activity became about 50% of the optimal value.

Divalent metal requirements

As has been reported with RNA polymerase from other organisms, the enzyme

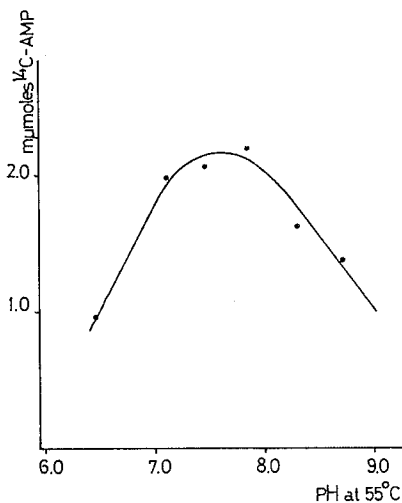


Fig. 1. The pH-activity curve of RNA polymerase. To the reaction mixture described in the text were added 70 μ g (by protein) of the enzyme fraction and 60 μ g of DNA. Incubation was done at 55°C for 20 min.

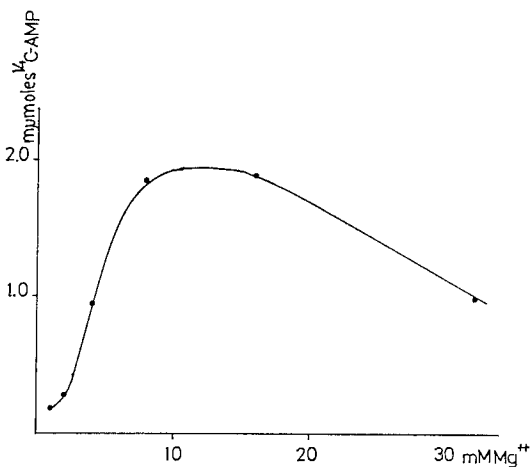


Fig. 2. Effect of magnesium on the activity of RNA polymerase. Reaction conditions were the same as in Fig. 1, except the concentration of Mg⁺⁺.

of *B. stearothermophilus* also required Mg^{++} for its RNA-synthesizing activity. In the absence of Mg^{++} no detectable activity was observed. Under the standard reaction conditions containing *B. stearothermophilus* DNA as template, the optimal Mg^{++} concentration was $1.0 \times 10^{-2} M$ (Fig. 2). In order to examine the effect of Mn^{++} on the activity, Mn^{++} was added to the reaction mixtures containing 4mM or 8mM Mg^{++} . In the presence of 4mM Mg^{++} , the maximum activity was obtained at 1.0mM (Fig. 3a). When the reaction mixture contained 8mM Mg^{++} , however, there was no optimal Mn^{++} concentration; the activity was rather inhibited at Mn^{++} concentrations higher than 1mM (Fig. 3b).

Time course of the RNA synthesis

Fig. 4 shows the time course of the ^{14}C -AMP incorporation into RNA at two different temperatures, 40°C and 55°C. The incorporation rate at 55°C was about 3 times of the rate at 40°C. By the addition of deoxyribonuclease (EC 3. 1. 4. 5), the incorporation was markedly inhibited. Elimination of CTP, GTP and UTP from the complete system also reduced the polymerization to the 0 time value. ^{14}C -ATP in above experiment was replaced by ^{14}C -GTP and very similar results were obtained.

The template requirement

Fig. 5 shows the template specificity of DNA's from different sources, which were prepared by the method described in the method section. About 25 μ g each of DNA was added to the standard reaction mixture and the ^{14}C -incorporation was measured as a function of the enzyme concentration. Under the standard reaction conditions, DNA of *B. stearothermophilus* was the most effective in priming

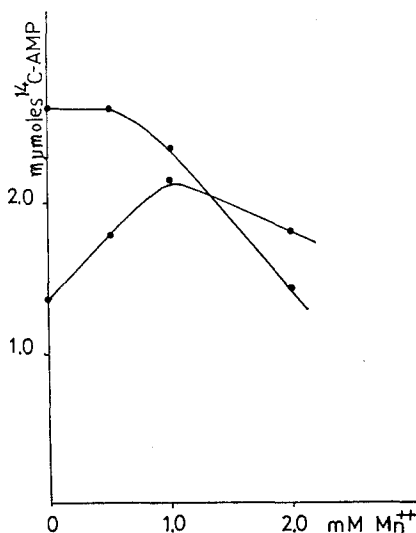


Fig. 3. Effect of manganese on the activity of RNA polymerase.
a) Reaction mixture containing 4mM Mg^{++} .
b) Reaction mixture containing 8mM Mg^{++} .

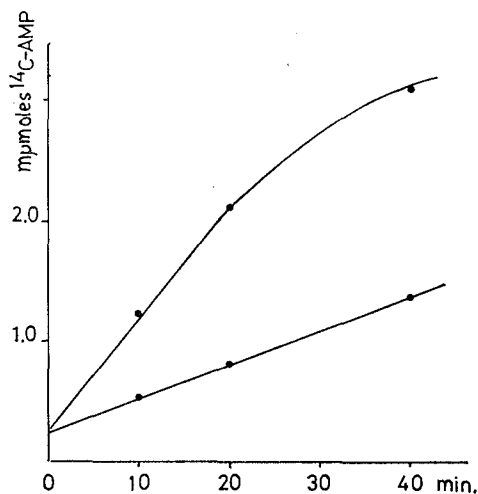


Fig. 4. Time course of the ^{14}C -AMP incorporation at 40°C and 55°C. Assay conditions were the same as in Fig. 1.

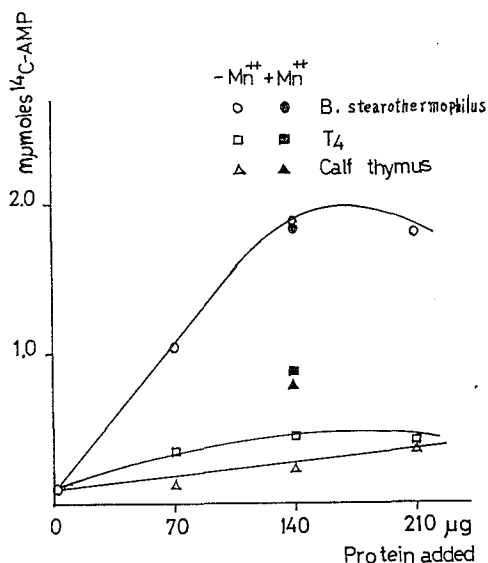


Fig. 5. The template activity of DNAs from different sources. Reaction mixtures contained 25 μ g of DNA and indicated amounts of the enzyme fraction. Incubation was done at 55°C for 20min.

RNA synthesis. It was interesting to note that such the template specificity was markedly modified by the addition of Mn⁺⁺. When 1 mM of Mn⁺⁺ was added to the reaction mixture, the template activity of T₄ and calf thymus DNA's increased to two and three times, respectively, whereas that of *B. stearothermophilus* was not affected. The similar change in the template specificity by Mn⁺⁺ was also observed with RNA replicase, which required bacteriophage RNA as template⁽¹¹⁾. The phenomenon appears to be very important to the template recognition mechanism of these RNA-synthesizing enzymes, though little is known of the molecular mechanism of the function of divalent metals.

As described above, RNA polymerase was partially purified from a thermophilic bacterium, and it was found that the enzyme was active at high temperature such as 55°C and also showed high affinity for homologous DNA as template in the presence of Mg⁺⁺. The enzyme appeared to be useful for the study of the specific interaction between RNA polymerase and the template DNA. Further purification and characterization of the enzyme are in progress.

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ABBREVIATIONS

DNA : deoxyribonucleic acid, RNA : ribonucleic acid, EDTA : ethylenediamine tetraacetate, GTP : guanosine triphosphate, ATP : adenosine triphosphate, CTP : cytidine triphosphate,

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UTP: uridine triphosphate, Tris: tris(hydroxymethyl) aminomethane.

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