A RAPID PROCEDURE FOR PURIFYING A RESTRICTION ENDONUCLEASE FROM *THERMUS THERMOPHILUS* (*Tth* I)

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1. Introduction

Restriction enzymes have proved to be a powerful tool for mapping genomes [1] and developing molecular cloning and DNA sequencing techniques [2]. A few restriction enzymes have been isolated from thermophilic bacteria [3], showing a high thermostability and resistance to protein denaturing agents. These properties could be useful to study DNA structure at higher temperatures. Looking for a stable enzyme with a new recognition sequence, we have isolated *Tth* I, an enzyme from the extreme thermophile *Thermus thermophilus* HB8 [4]. This thermostable enzyme turned out to be an isoschizomer of *Taq* I, which recognizes the sequence 5′-TCGA-3′ [5]. *Thermus thermophilus* is a very convenient source for purifying this enzyme since this bacterium does not produce the pigmented 'slime' described in *Thermus aquaticus* [6], which interferes with the purification of *Taq* I [5,7] and other enzymes [8,9]. Furthermore, *Tth* I was readily released by osmotic shock, which allowed us to develop a simplified, rapid two-step procedure to obtain an enzyme preparation free of contaminating nucleases. We report here the purification method and some of the properties of *Tth* I.

2. Materials and methods

2.1. *Source of materials*

Thermus thermophilus HB8 strain was obtained from American Type Culture Collection (ATCC 27634). Cells were grown at 75°C with vigorous shaking and were harvested by centrifugation before the end of the log phase of growth. The culture media contained 4 g yeast extract, 6 g polypeptone and 3 g NaCl per liter. Agarose (1%) (Seakem) vertical slab gels were run in 50 mM Tris–HCl, 40 mM sodium acetate and 1 mM EDTA (pH 8.0). To analyze small DNA fragments, acrylamide (Bio-Rad) gels were run in 90 mM Tris–HCl, 70 mM boric acid and 2.5 mM EDTA (pH 8.3). Gels were stained with ethidium bromide (1 μg/ml) and photographed under ultraviolet light. *Taq* I restriction enzyme was obtained from BRL.

2.2. *Preparation of DNAs*

λ DNA was prepared from λ-lysogenic *E. coli* strain 594λ*c*I857Sam7 (generously provided by H. Murialdo) as in [10]. PM2 phage DNA, *Col* E1 and pBR322 plasmid DNAs were prepared using standard procedures.

2.3. *Assay for restriction enzymes*

Digestions with either *Tth* I or *Taq* I were carried out in 20 mM Tris–HCl (pH 8.0), 5 mM $MgCl_2$, 10 mM 2-mercaptoethanol and 50 mM NaCl. DNA (~1 μg) dissolved in 10 mM Tris–HCl (pH 8.0). EDTA (1 mM) was added to 20 μl final vol. The samples were incubated for 60 min at 60°C and the reactions were halted with 10 μl solution containing 20% glycerol, 1% SDS, 50 mM EDTA and 0.05% bromophenol blue.

3. Results and discussion

3.1. *Purification steps*

The isolation procedure involves osmotic shock treatment [11]. Bacterial cells (10 g) were washed twice with 200 ml of a buffer containing 10 mM Tris–HCl (pH 7.8) and 30 mM NaCl, resuspended in 200 ml 33 mM Tris–HCl buffer (pH 7.8) containing 3 mM EDTA and 20% sucrose and stirred at room temperature for 20 min. After centrifugation at 16 000 × *g* for 5 min, cells were resuspended in

150 ml 0.5 mM $MgCl_2$, stirred at room temperature for 10 min and centrifuged at 16 000 × *g* for 5 min. The supernatant was made 20 mM Tris–HCl (pH 7.5), 4 mM 2-mercaptoethanol, 1 mM EDTA and 20% glycerol (buffer A) and applied to a DEAE-cellulose column (1 × 15 cm). The enzyme was eluted with a 400 ml linear gradient from 0–0.5 M NaCl in buffer A. The active fractions, eluting between 0.13–0.18 M NaCl were loaded onto a 5 ml phosphocellulose column pre-equilibrated with buffer A. The enzyme was eluted stepwise using 6 ml of buffer A containing 0.2, 0.3, 0.4, 0.5 and 0.6 M NaCl. The activity eluted at 0.6 M NaCl was concentrated by dialysis against 30% polyethylene glycol dissolved in buffer A. This final fraction was 0.5 ml and 0.65 mg protein/ml. At 2 μg, this enzyme preparation produced complete digeston of 1 μg λDNA in 30 min at 60°C. If the incubation time was extended for 12 h, the same sharp band pattern is observed in polyacrylamide gel electrophoresis. *Tth* I restriction enzyme can thus be obtained in 1 day free of contaminating nucleases. Since *Tth* I is released by osmotic shock, this enzyme is most probably located in the periplasmic space [12].

3.2. *Properties of* Tth *I restriction enzyme*

Searching for the recognition sequence of *Tth* I, we found that the pattern of restriction fragments produced after digestion with various DNAs was identical to that obtained with the *Taq* I enzyme (fig.1). Therefore, *Tth* I and *Taq* I endonucleases are isoschizomers.

The effect of incubation temperature and temperature stability of *Tth* I are shown in fig.2. Optimal incubation temperature is 60°C, showing very little activity at 37°C and incomplete digestion at 50°C or 70°C. Figure 2 also shows the thermostability of *Tth* I at 80°C. The enzyme was pre-incubated at this temperature for different time intervals and then assayed under standard conditions. A 10 min pre-incubation period does not affect *Tth* 1 activity, although after 1 h at 80°C the enzyme is partially inactivated.

Tth I is neither affected by NaCl up to 0.3 M, nor by changes in pH over 7.5–8.5. In contrast to other restriction enzymes [13], $MnCl_2$ at 1.5 mM can replace efficiently $MgCl_2$ without any change in enzyme specificity.

We have recently isolated a plasmid from *Thermus thermophilus* and are currently investigating if *Tth* I is a plasmid-coded enzyme.

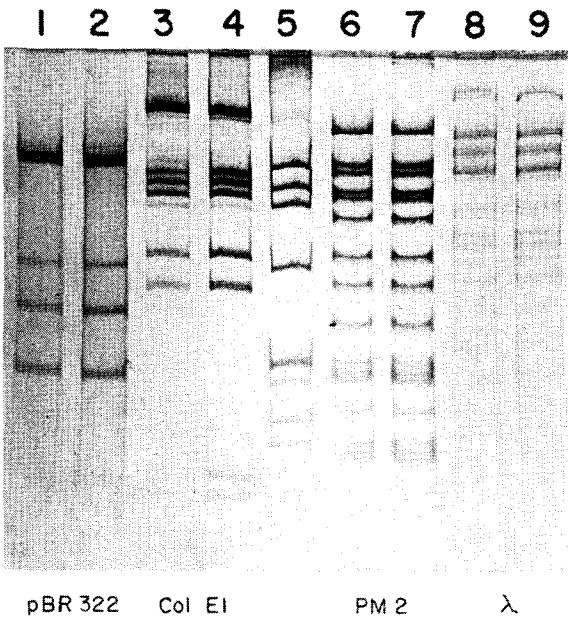

Fig.1. Digestion pattern of various DNAs with *Tth* I and *Taq* I. The DNA fragments produced were analyzed in a 7% acrylamide gel. Lanes 1,3,6,8: *Tth* I digestion. Lanes 2,4,7, 9: *Taq* I digestion. Lane 5 contains molecular weight standards obtained by treatment of φXRfi DNA with *Hae* III restriction enzyme.

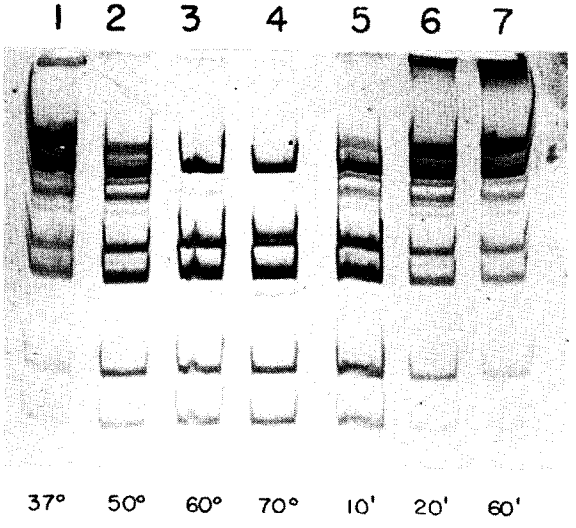

Fig.2. Effect of *Tth* I on *Col* EI DNA under different experimental conditions. The DNA fragments obtained were separated in a 5% acrylamide gel. Lanes 1–4: assays carried out at the temperatures indicated. Lanes 5–7: *Tth* I pre-incubated at 80°C for the indicated time intervals before assaying under standard conditions (see section 2).

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References

[1] Nathans, D. and Smith, H. O. (1975) Ann. Rev. Biochem. 44, 273–293.
[2] Roberts, R. J. (1976) CRC Crit. Rev. Biochem. 4, 123–164.
[3] Roberts, R. J. (1978) Gene 4, 183–193.
[4] Oshima, T. and Imahori, K. (1974) Int. J. Syst. Bacteriol. 24, 102–112.
[5] Sato, S., Hutchison, C. A. and Harris, J. I. (1977) Proc. Natl. Acad. Sci. USA 74, 542–546.
[6] Broch, T. D. and Freeze, H. (1969) J. Bacteriol. 98, 289–297.
[7] Bickel, T. A., Pirrotta, V. and Imber, R. (1977) Nucleic Acids Res. 4, 2561–2572.
[8] Sato, S. and Harris, J. I. (1977) Eur. J. Biochem. 73, 373–381.
[9] Stellwagen, E., Cronlund, M. M. and Barnes, L. (1973) Biochemistry 12, 1552–1558.
[10] Becker, A. (1970) Biochem. Biophys. Res. Commun. 41, 63–70.
[11] Heppel, L. A. (1971) in: Structure and Function of Biological Membranes (Rothfield, L. C. ed) pp. 224–251, Academic Press, New York.
[12] Smith, D. I., Blattner, F. R. and Davis, J. (1976) Nucleic Acids Res. 3, 343–353.
[13] Hsu, M. and Berg, P. (1978) Biochemistry 17, 131–138.