

Cloning and Expression of *Thermus Aquaticus* DNA Polymerase Gene, Using a Thermo-Inducible Expression Vector

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ABSTRACT

DNA polymerase gene from *Thermus aquaticus* strain YT1 was amplified using VENT™ DNA polymerase and cloned under the control of *X.P_R* promoter and expression was induced by a shift in temperature. The culture was then sonicated, and after centrifugation the lysate was treated with polyethyleneimine followed by a salting-out step. Finally the protein was precipitated with ammonium sulfate and fractionated by gel filtration. The resulting enzyme preparation was stable and active. *Iran. Biomed. J* 2: 79-82, 1998

Keywords: *Taq* DNA polymerase, PCR, Thermal induction

INTRODUCTION

A thermostable DNA polymerase was originally purified and characterized from *Thermus aquaticus* (*Taq*) strain YT-1 [1] which proved to be suitable and vital for *in vitro* amplification of DNA fragments via the polymerase chain reaction [2]. The low yield of enzyme by its native host (0.01-0.02% of the total protein) [3] and the increased demand for thermostable polymerases like *Taq* DNA polymerase for various molecular biology application triggered the search for methods increasing the yield and activity. Therefore recombinant DNA technology was used to isolate and express the gene in *Escherichia coli* [3, 4]. Both regulation of expression and purification methods are considered important factors for production of recombinant proteins. Various methods have been reported for production of *Taq* DNA polymerase in *E.coli* [5, 6] yielding homogenous enzyme with high activity. In all these systems isopropylthio-β-D-galactoside (IPTG) has been used as the chemical inducer of expression. However in the present study temperature shift was employed for induction of *Taq* DNA polymerase gene cloned under the control of X promoter which provides tight control of expression and eliminates the need for an inducing metabolite.

MATERIALS AND METHODS

Bacterial strains and Plasmids. *Thermus aquaticus* YT-1 (DSM Germany no. 625 kindly provided by Mr. A. R. Gholamei) was used to obtain thermostable DNA polymerase gene. *E.coli* strain SG (*Agal dlac Ion⁻ 146:Tn10 1117 leu sup⁺ rec⁻Tet^R*) was the host strain and vectors pGEMEX-1 (Promega company) and pACT-7 (Dr. V.O. Rechinsky) were used for cloning and expression.

PCR and Cloning. The genomic DNA was isolated from *T. aquaticus* and used as template for amplification by PCR [4]. The primers were synthesized and kindly provided by Dr. B.K. Chernov, (V.A. Engelhardt Institute of Molecular Biology, USSR Academy of Science, Moscow.). The amino terminal primer was: 5'-TCACCATATGAGGGG GATGCTGCCCTCTTTGAG creating a unique underlined *NdeI* site and the carboxy terminal was: 5'-CATAGCGAATTCTATCACTCCTTGGCGGAGAGCC creating a unique underlined *EcoRI* site. The PCR condition was as follows: 94°C 1 min. and 72°C 2 min. for 30 cycles, Vent DNA polymerase (New England Biolab) was used for amplification of product (Figure 1). All basic recombinant DNA techniques were carried out according to standard methods [7]. The PCR product was digested with *NdeI* and *EcoRI* and cloned in pGF, MEX-1. The resulting construct

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was cut with *Bgl* II and *Sph* I, the fragment thus obtained contained *Taq* DNA polymerase gene and it was subcloned in an expression vector pACT7 which is digested with *Bat* III and *Sph* I. The resulting construct was designated pACTaq (Figure 2) and was used to transform SG cells. The recombinant clones containing *Taq* DNA polymerase gene were screened on LB plate containing chloramphenicol (20 µg/ml), and processed for plasmid extraction, 3 suitable clones were selected and digested with Hind III, PstI and Pvu II (Figure 3). Restriction enzymes are all from Gibco-BRL. After confirmation with restriction map analysis one of the clone was chosen for expression.

Expression and Purification of *Taq* DNA Polymerase. *Taq* DNA polymerase was purified from 200 ml tryptic soy broth seeded with an overnight culture of 5 ml of transformed SG bacteria grown in the presence of chloramphenicol (20 µg/ml) and grown to optical density (OD₆₀₀ nm) of 0.3 at 32°C. The induction was performed by immediate shift of temperature from 32 to 42°C and the culture was allowed to grow for an additional 4-5h. Then they were pelleted by centrifugation and dissolved in buffer A (10 mM Tris-HCl, pH 7.6, 20 mM NaCl, 5 mM 13-mercaptoethanol) containing 1 mg/ml lysozyme and left at room temperature for 15 min. They were sonicated after the addition of 100 µl 0.5M EDTA (pH 7.9). The lysate was diluted with 1% buffered-Triton X-100 and centrifuged at 30000 g for 1h at 4°C. Polyethylimine (PEI 10%) was added to the supernatant to a final concentration of 0.07%. The mixture was vortexed and left for 15 min. at room temperature and then centrifuged at 12000 g for 1/2h. The pellet was dissolved in buffer B (5 mM Tris-acetate, 50 mM NaCl, 1 mM 13-mercaptoethanol) and kept at 4°C for 15 min. The mixture was centrifuged again at 12000 g for 5 min. and the pellet was extracted with buffer C (40 mM Tris-acetate, 0.5M NaCl, 1 mM, 13-mercaptoethanol), the supernatant was collected and the pellet was washed with buffer B and the extraction was repeated. Ammonium sulfate was added to the supernatant to a final concentration of 85% saturation and after the centrifugation the pellet was dissolved in buffer D (50 mM Tris-acetate, 0.1M NaCl, 4% glycerol) and either was boiled for 5-10 min. and then briefly centrifuged and the supernatant was used as a source of enzymes or/ before boiling was loaded on Sephacryl S-300 column and the enzyme was eluted with 150 ml linear gradient of 0-0.1M

NaCl. Fractions containing DNA polymerase activity were pooled and dialyzed against buffer E (40 mM Tris-acetate, 50 mM NaCl, 1 mM 0-mercapto-ethanol, 50% glycerol) and stored at 20°C. The protein level was determined spectrophotometrically. SDS-PAGE [8] was used for characterization of the protein (Fig. 4).

RESULTS AND DISCUSSIONS

All data thus far reported on the expression of *Taq* DNA polymerase in *E. coli* [3-6] had used metabolite induction. In this study *Taq* DNA polymerase gene was cloned under the control of *Xpr*R promoter in expression vector pACT-7 in which temperature shift rather than chemical is used for induction, a system resembling the vector pCQV2 [9].

It has been stated that increase in temperature may affect the amount of properly folded proteins [10] and also stimulates the production Lon protease and other heat shock proteins [11]. The purification scheme followed in this study consisted of bacterial lysis and precipitation of proteins along with nucleic acids by addition of PEI (10%). (The concentration

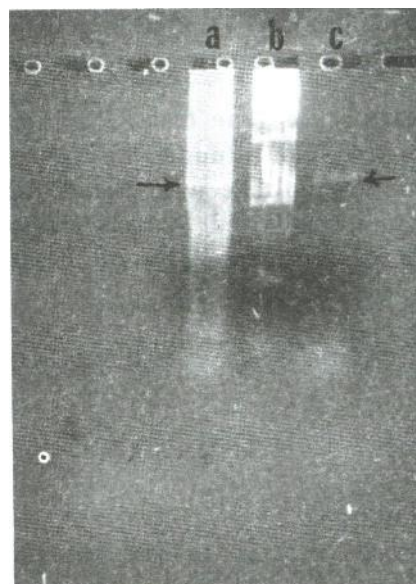


Fig. 1. PCR product using Vent DNA polymerase; Lane a, amplification of undigested genomic DNA from *Thermus aquaticus* strain (2.5 kb PCR product indicated by arrow). Lane b, MW. marker k DNA/Hind III fragments (23130, 9416, 6557, 4361, 2322, 2027, 564 and 125 bps). Lane c, amplification of digested genomic DNA/EcoRI from *Thermus aquaticus* strain (2.5 kb PCR product indicated by arrow).

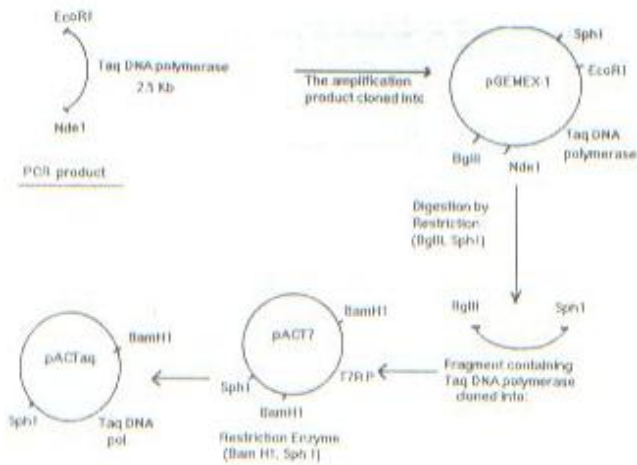


Fig 2. Schematic presentation of different stages in construction of pACTaq, which contains Taq DNA polymerase gene.

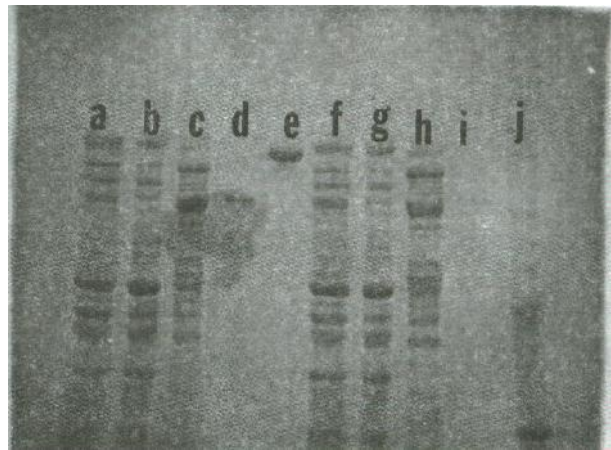


Fig. 4. SDS-PAGE analysis. From left to right, shows the protein profile of two clones expressing Taq DNA polymerase; (a), crude lysate, (b), after PEI precipitation, (c), extracted protein, (d), after heating, (e), MW 97.4 kDa (MW-SDS-200, Sigma). Lanes f-i: same as above for another clone. Lane j, MW-SDS-200 (Sigma).

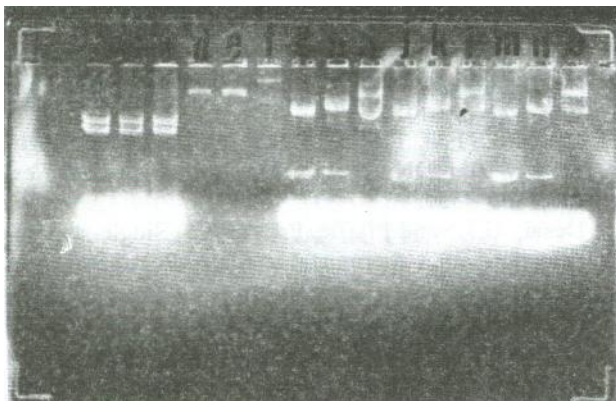


Fig 3. Restriction map analysis of clones containing Taq DNA polymerase gene. From left to right: Lanes a to c: 3 identical clones (pACTaq) digested with Pvu II. Lanes d to f: vector without insert (pACT7) digested with Hind III/Pst I, digested with Hind III, undigested plasmid respectively. Lanes g to o: three identical clones (pACTaq) digested with Hind III/Pst I, digested with Hind III, undigested plasmid.

of PEI was found to be critical since its high concentration makes the extraction of protein from the resulting pellet difficult. Extraction was performed with high salt concentration buffer C (500 mM) and finally the protein was precipitated efficiently with addition of ammonium sulfate. In our study it was shown that treating the lysate either by boiling or the use of simple chromatographic step can be employed alternatively and efficiently, although various methods using affinity, ion-

exchange and gel filtration alone or in combination have been reported [1, 4, 12]. The use of clear lysate without any further purification steps has also been reported [5, 6]. In this study also the lysate was boiled and then centrifuged, the clear lysate was still stable and capable of polymerization at high temperature. But it seems gel filtration used in this study can also be a simple scheme, which enables enzyme to be more stable over longer time, due to sieving effect of gel filtration, which helps to obtain enzyme with higher purity.

In the present study enzyme activity was assayed against commercially available Taq DNA polymerase from Gibco-BRL by titration using PCR (Figure 5). The concentration of enzyme obtained (unit/11) was comparable with the commercial enzyme, whenever the template M13mp18 and primers of high purity and specificity were used but the superiority of commercial enzyme was evident when the template was either genomic or not purified enough and also when primers were not very pure (data not shown). In conclusion the expression system and the purification scheme used in this study yields enough enzyme of research quality and provides a ready source of this essential enzyme for molecular biology research. Moreover, since thermostable proteins are finding increasing demands, thermo-inducible vectors such as the one used in this study might be worthy of more attention.

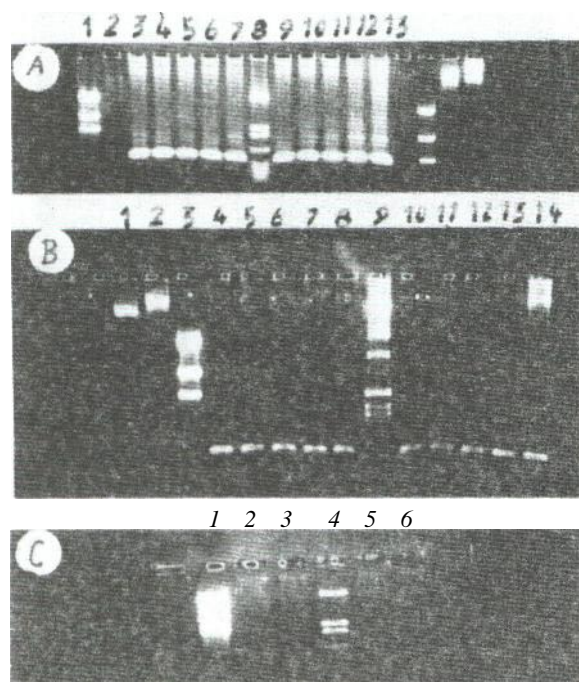


Fig. 5. Activity-comparison of purified *Taq* DNA polymerase enzyme by titration in PCR assay. The pooled fractions after dialysis was diluted 1:10, 1:100, 1:200, 1:400, 1:800 to 1:25600 with 1x PCR buffer for activity assay. One ul of diluted enzyme was added to 20 ul reaction mixture using M13mp18 as template and universal forward primer for single-round primer extension and both universal and reverse primers for PCR amplification of 35 cycles; 1 min at 94°C, 1 min at 55°C and 1.5 min at 72°C. Panel A : Lanes A₁ and A₂ containing molecular weight marker (MW), Lane A₂ blank, Lanes A₃-A₆ showing enzyme dilutions: 1/10, 1/100, 1/200, 1/400, Lane A₇ undiluted of commercial enzyme. Lanes A₉ to A₁₂ the same dilution with purified enzyme and A₁₃ undiluted enzyme. Panel B : Lane B₁ and B₂ single and double stranded M13, B₃ and B₉ molecular weight marker (MW) B₄ to B₈ undiluted, 1/800 1/1600 1/3200 and 1/6400 of commercial enzyme, B₁₀ to B₁₄ the same dilution with purified enzyme. Panel C: Lane C₁ and C₄ molecular weight marker (MW), C₂ and C₃ 1/12800, 1/25600 of commercial enzyme, C₅ and C₆ same dilution with purified enzyme (in these two last dilution the bands are very faint and not clearly visible in figure).

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