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

Saeid Bouzari* and Veladimir O. Rechinsky

Molecular Biology Unit, Pasteur Institute of Iran, Tehran 13164, I.R. Iran

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 tern perature. 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. Biorned. 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 p - lymerase 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-I3-D-galactoside (IPTG) has been used as the chemical inducer of expression. However in the present study temper - ture 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 aquat - cus 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 e'pression.

PCR and Cloning. The genomic DNA was isolated from *T. aquaticus* and used as template for amplification by PCR [4]. The primers were synth sized 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'-TCACCATATGA GGGG GATGCTGCCCCTCTTTGAG creating a unique underlined *NdeI* site and the carboxy terminal was: 5 '-CATAGCGAATTCTATCACTCCTTGGCGGA GAGCC 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 po - lymerase (New England Biolab) was used for amplification of product (Figure 1). All basic recom binant DNA techniques were carried out according to standard methods [7]. The PCR product was di - gested with *NdeI* and *EcoRI* and cloned in pGF, MEX-1. The resulting construct

*Corresponding Author;

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was cut with *Bgl II* and *Sph 1*, the fragment thus obtained contained *Taq* DNA polymerase gene and it was subcloned in an expression vector pACT7 which is digested with *Batnal* and *Sphl*. 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 tg/ml), and processed for plasmid extraction, 3 suitable clones were selected and digested with Hind HI, 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 e - pression.

Expression and Purification of Taq DNA P - lymerase. 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 (2011g/m1) and grown to optical density ($0D_{600}$ 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-HC1, pH 7.6, 20 mM NaCl, 5 mM 13-mercaptoethanol) containing Img/m1 lyzo- syme and left at room temperature for 15 min. They were sonicated after the addition of 100 µ10.5M EDTA (pH 7.9). The lysate was diluted with 1% buffered-Triton X-100 and centrifuged at 30000 g for lh at 4°C. Polyethyleneimine (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 NaC1, 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 NaC1, 1 mM, 13-mercapto-ethanol), the supernatant was collected and the ppllet was washed with buffer B and the extraction was repeated. Ammonium sulfate was added to the supernatant to a final concentration of 85% satura- tion and after the centrifugation the pellet was dis- solved in buffer D (50 mM Tris-acetate, 0.1M NaC1, 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 NaCI. 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 spectro- photometrically. SDS-PAGE [8] was used for char - acterization of the protein (Fig. 4).

RESULTS AND DISCUSSIONS

All data thus far reported on the expression of ***Taq*** DNA polymerase in ***E.*** *co/i* [3-6] had used me- tabolite induction. In this study ***Taq*** DNA p - lymerase gene was cloned under the control of *XpR* 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 purifi cation scheme followed in this study consisted of bacterial lysis and precipitation of proteins along with nucleic acids by addition of PEI (10%). (The concentration

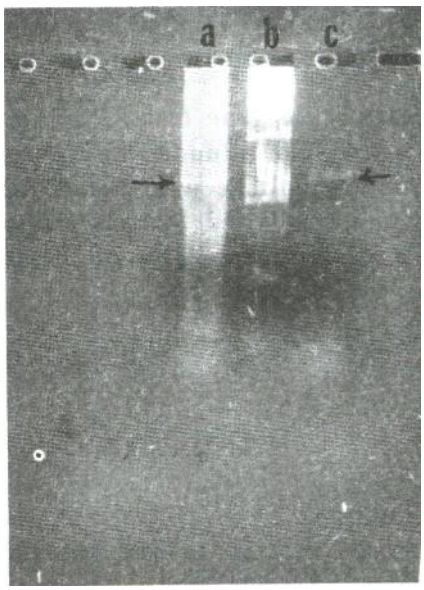

Fig. 1. PCR product using Vent DNA polymerase; Lane a, amplification of undigested genomic DNA from *Thermus aquaticus* strain (2.5 kb PCR product indicated by arrow). Lane b, MW. marker k DNA/Hind III fragments (23130, 9416, 6557, 4361, 2322, 2027, 564 and 125 bps). Lane c, amplification of digested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

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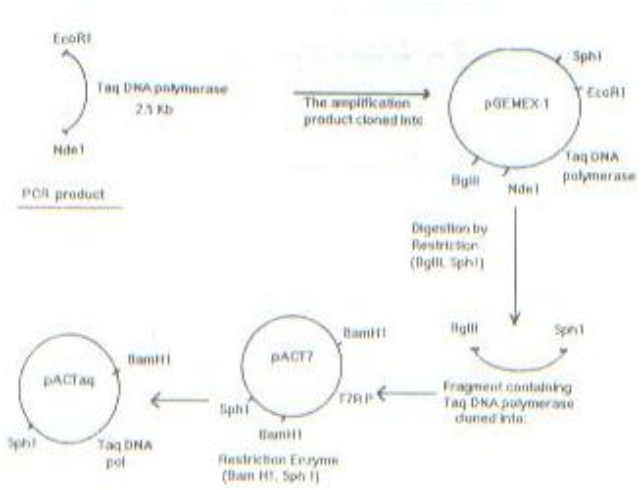

Fig 2. Schematic presentation of different stages in construction of pACTaq, which contains Taq DNA polymerase gene.

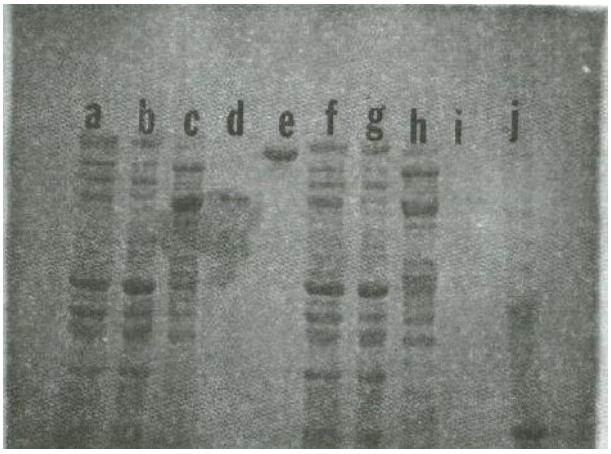

Fig. 4. SDS-PAGE analysis. From left to right, shows the protein profile of two clones expressing Taq DNA polymerase; (a), crude lysate, (b), after PEI precipitation, (c), extracted protein, (d), after heating, (e), MW 97.4 kDa (MW-SDS-200, Sigma). Lanes f-i: same as above for another clone. Lane j,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

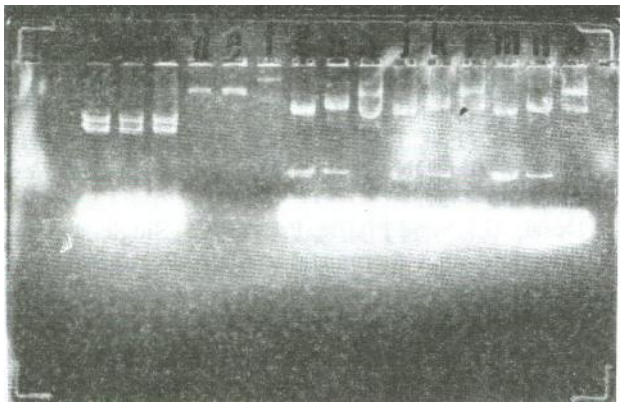

Fig 3. Restriction map analysis of clones containing Taq DNA polymerase gene. From left to right: Lanes a to c: 3 identical clones (pACTaq) digested with Pvu II. Lanes d to f: vector without insert (pACT7) digested with Hind III/Pst I, digested with Hind III, undigested plasmid respectively. Lanes g 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 chr - matographic step can be employed alternatively and efficiently, although various methods using affinity, ion-exchange and gel filtration alone or in combi nation 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 p - lymerase from Gibco-BRL by titration using PCR (Figure 5). The concentration of enzyme obtained (unit/1_11) was comparable with the commercial enzyme, whenever the template M13mP1 8 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 puri - fled 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.

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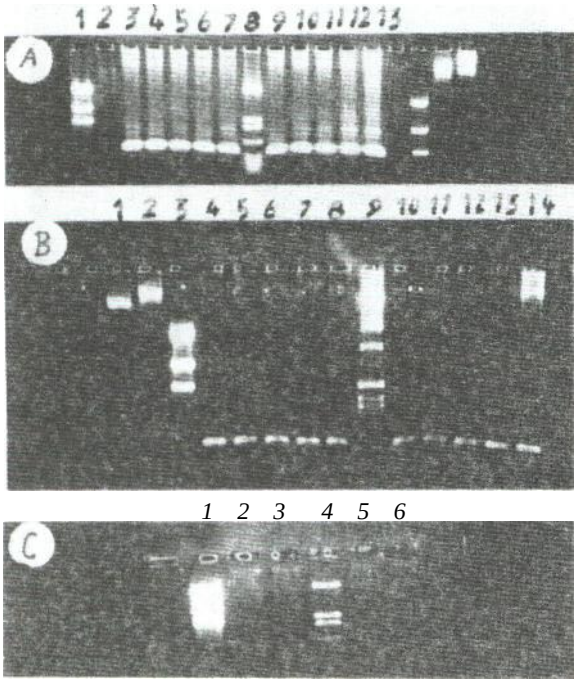

Fig. 5. Activity-comparison of purified *Taq* DNA polymerase enzyme by titration in PCR assay. The pooled fractions after dialysis was diluted 1:10, 1:100, 1:200, 1:400, 1:800 to 1:25600 with lx PCR buffer for activity assay. One ul of diluted enzyme was added to 20 ul reaction mixture using M13mp18 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 A_I and A_g containing molecular weight marker (MW), Lane A_2 blank, Lanes A_3-A_6 showing enzyme dilutions: 1/10,1/100,1/200, 1/400, Lane A_7 undiluted of commercial enzyme. Lanes A_9 to A_{l2} the same dilution with purified enzyme and A_{13} undiluted enzyme. Panel B : Lane B_1 and B_2 single and double stranded M13, B_3 and B_9 molecular weight marker (MW) B_4 to B_8 undiluted, 1/800 1/1600 1/3200 and 1/6400 of commercial enzyme, Bic) to B_{14} the same dilution with purified enzyme. Panel C: Lane C_I and C_4 molecular weight marker (MW), C_2 and C_3 1/12800, 1/25600 of commercial enzyme, C_5 and C_6 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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