Cloning and Expression of *Thermus aquaticus* DNA polymerase in *Escherichia coli*

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Abstract
Thermostable DNA polymerase gene from *Thermus aquaticus* was cloned into constructed Taq from Thermus a Qaticus (pTTQ) plasmid using *Eco*RI and *Sal*I sites with subsequent transformation in *Escherichia coli* strain (TOP10). The use of Isopropyl-β-D-thiogalactopyranosid (IPTG) as inducer of interested gene expression under control of the *lac* promoter was investigated. The optimization of enzyme induction by IPTG was determined at shake flask level to be 0.52mM at exponential growth phase. Enzyme preparation was performed by lysis the cultured cells. Afterwards, the cell suspension was incubated at 75°C to denature all heat sensitive proteins in the cell suspension that have been removed by subsequent centrifugation. Finally, the clarified supernatant containing heat resistant *Taq* DNA polymerase was collected and stored at -80°C. The activity of enzyme was compared with commercial *Taq* DNA polymerase, which remained when stored in buffer containing 50% glycerol, at -20°C. The purified enzyme had a molecular weight of 94 KDa, as estimated by SDS-PAGE and yielded appropriate enzyme activity comparing to the commercial *Taq* DNA polymerase.

Keywords: *Taq* DNA Polymerase, *E. coli*, expression, *Thermus aquaticus*

Introduction

Thermostable DNA polymerase is a very important enzyme for molecular biological studies such as DNA amplification and DNA sequencing by the polymerase chain reaction (PCR) [1, 2]. Most of the thermostable DNA polymerases have been isolated from *Thermus aquaticus*, a thermostable bacterium, known as *Taq* polymerase. *Taq* DNA polymerase is an enzyme obtained from a heat stable bacterium called *T. aquaticus* having a molecular weight of about 6.6×10^4–9.4×10^4 Daltons [3]. *T. aquaticus* is a bacterium that lives in hot springs and hydrothermal vents [4]. *Taq* polymerase was identified as an enzyme able to withstand the protein-denaturing conditions (high temperature) required during PCR [5]. Therefore it replaced the DNA polymerase from *E. coli* originally used in PCR [6]. Taq's temperature optimum for activity is 75-80°C, with a half-life of 9 minutes at 97.5°C, and can replicate a 10^3 base pair strand of DNA in less than 10 seconds at 72°C [7].

Taq DNA polymerase catalyzes the incorporation of dNTPs into DNA. It requires a DNA template, a primer terminus, and the divalent cation Mg++. *Taq* polymerase contains a polymerization dependent 5'-3' exonuclease activity. It does not have a 3'-5' exonuclease and thus no proof reading function. Despite this, the enzyme synthesizes DNA *invitro* with reasonable fidelity [8]. Use of the thermostable Taq polymerase eliminates the need for having to add new enzyme to the PCR reaction during the thermocycling process. A single closed tube in a relatively simple machine can be used to carry out the entire process. Thus, the use of Taq polymerase was the key idea that made PCR applicable to a large variety of molecular biology problems concerning DNA analysis [5].

The Taq DNA polymerase isolated from *T. aquaticus* was the first characterized thermostable enzyme but more than 50 DNA polymerase genes have been cloned and sequenced from various organisms including thermophiles by PCR cloning technique, whereby the gene encoding this enzyme was cloned into the expression vectors that produce recombinant *Taq* polymerase gene has facilitated for this enzyme production [3]. The recombinant *Taq* DNA polymerase expressed in *E. coli* shows identical characteristics to native *Taq* from *T. aquaticus* with respect to activity, specificity, thermostability and performance in PCR [9]. However, the *lac* promoter and its derivatives are widely employed for the purposes mentioned above, and in most cases, IPTG is used as inducer for foreign gene expression [4, 5]. Our goal, in the present study, is the cloning and expression of recombinant *Taq* DNA polymerase in the *E. coli* for performance in PCR.

Materials and Methods

Molecular cloning of the gene for Taq DNA polymerase

Genomic DNA of *T. aquaticus* and plasmid DNA were isolated by a method adapted from Sambrook [7]. A 2.6 Kb fragment containing the whole *T. aquaticus* DNA polymerase gene was prepared by PCR amplification [2] with the *T. aquaticus* genomic DNA using primers forward-primer 5' - CGG AAT TCT GAG GAG GTA ACA TGA GGG -3' and the reverse-primer sequence 5'-CGT CGA CTA GAT CAC TCC TTG GCG GAG AG -3' which created the underlined unique *Eco*RI and *Sal*I restriction sites respectively at each end of the amplified DNA fragment. The primer sequences were adopted as described [9]. The fragment was ligated into the expression vector pET (*invitrogene*) that had been digested before with *Eco*RI and *Sal*I (Sigma), giving a closed circular fusion molecule (The constracted vector have been called pTTQ. The ligate was transformed into competent *E. coli* strain, TOP 10 (Sinagen) by $CaCl_2$ (Sigma) using heat shock method at 42°C for 45 seconds [8].

Culture and expression conditions

The recombinant *E. coli* was cultured in 10 ml of Lauria Bertaini (LB) broth (Merck) overnight at 37°C containing 100µg/ml ampicillin (Merck) as seed culture. The LB medium containing 100µg/ml ampicillin was inoculated with 1% of seed culture (250ml of LB for shake flask system) and grown at 37°C. The expression of recombinant protein was induced by 0.52mM IPTG (Sigma) to the growing culture at an OD_{600} of 0.6-0.8. The culture was continued overnight. No addition of inducer was used as negative control experiment [10].

Enzyme extraction and purification

The cells were harvested by centrifugation at high speed, washed, and then resuspended in buffer A [(1 mM EDTA; Sigma), 50 mM Tris–HCl (pH: 8.0) (Sigma), 50 mM Glucose (Merck)] and buffer B [1 mM EDTA, 50 mM Tris–HCl (pH: 8.0), 50 mM Glucose, 4 mg/ml Lysozyme, Sigma] to a twentieth of the culture volume. The cells were then lysed by adding 15ml of lysis buffer [10 mM Tris–HCl (pH: 8.0), 0.1 mM EDTA, 0.5% Tween 20, Merck, 0.5% Nonidet P40, Sigma, 50 mM KCl, Sigma, 1.0 mM PMSF, Sigma**]**. The suspension was incubated for 1h at 75°C. Cellular debris was removed by centrifugation at 1.8×10^4 rpm for 10 min and the clarified supernatant was stored in the storage buffer as described [9].

Enzyme assay and protein determination
Recombinant protein was analyzed by SDS-PAGE [8]. The activity of the enzyme was determined by using a PCR amplification reaction with titration against a commercial *Taq* preparation (Roche). Human genomic DNA extracted from whole blood by DNA extraction kit (Genfanavaran) was used as template for subsequent amplification reactions. The PCR was amplified with specific product of 250bp fragment. To improve the enzyme activity, RD-buffer (Recombinant Detergent; self created name) containing Tris-HCl pH=8.8; 1mg/ml Bovian Serum Albumin (BSA); mercaptoethanol 0.1mM and ammonium sulfate (0.160mM) was used in the PCR and the result was compared with standard PCR buffer.

Results

The full length of *Taq* polymerase gene was first PCR amplified and inserted into pTTQ vector. The recombinant plasmid was transformed into *E. coli*, and extracted from the cell culture. After digestion with *Eco*RI and *Sal*I restriction enzyme, the *Taq* polymerase gene was gel purified and inserted into an expression vector constructed as pTTQ vector. Following the plasmid transformation, the expression of *Taq* polymerase was performed in 250ml in a shake flask by induction with different concentration IPTG that optimized concentration shown at 0.52mM. The induction was performed at the exponential phase. The enzyme was stored in 50% glycerol. The partial purification of the enzyme was performed through short boiling time and subsequent incubation at 75°C [2, 7-10]. The partial purified of the enzyme was monitored by SDS-PAGE (Fig. 1).

To estimate the enzyme activity, different fragments were PCR amplified with recombinant *Taq*. The PCR products suggested that 2µl of purified enzyme yields comparable results with 0.5µl of commercial *Taq* polymerase, which might be the resulting from either the existence of inhibitory agents in the purified enzyme solution or a lower enzyme concentration (Fig. 2).

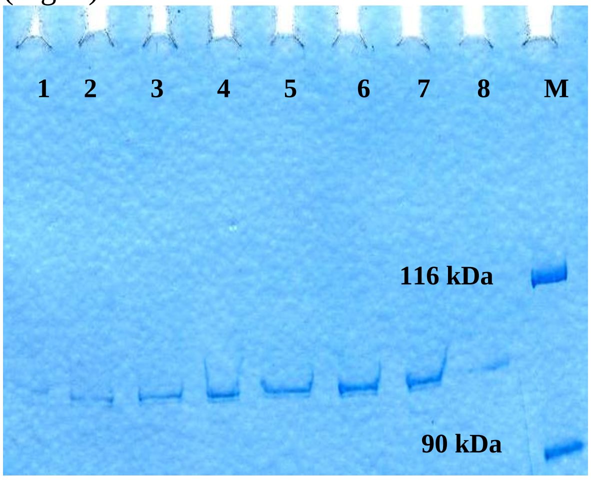

Fig. 1: SDS-PAGE analysis of *Taq* DNA polymerase. Lane 1:0.5µl purified Taq, lane 2: 1µl Taq, lane 3:2µl Taq, lane 4:3µl Taq, lane 5: 4µl Taq, lane 6:5µl Taq, lane 7:6µl Taq, lane 8: 0.5µl (2.5 units) commercial Taq DNA polymerase, M: protein size marker

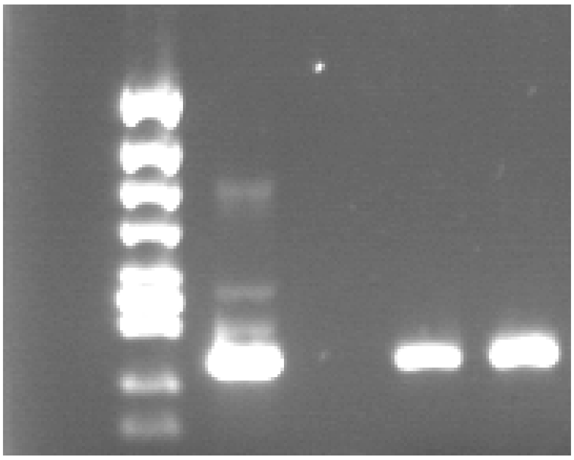

Fig. 2: Enzyme activity assay. Different PCR were performed with 1) 0.5µl commercial Taq, compared with 2) 0.5µl, 3) 1µl and 4) 2µl expressed and purified Taq DNA polymerase. M) DNA size marker

It was also performed in a PCR with a buffer (called RD buffer) obtained improved ingredients as ammonium sulfate and BSA [6, 9, 10]. Under new conditions a 0.5µl of enzyme was able to yield comparable amount of PCR products as 2.5 units (0.5µl) of commercial enzyme (Fig. 3).

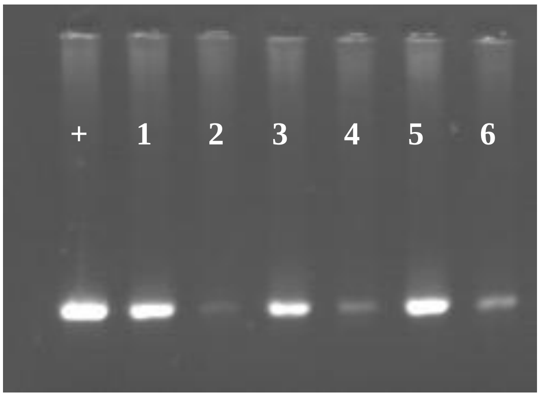

Fig. 3: Improved effect of RD - buffer in the PCR. Lanes 1, 3 & 5 show the reactions performed with 0.5µl, 1µl & 2µl purified enzyme and RD buffer respectively. Lanes 2, 4 & 6 indicate the PCR under same conditions performed with standard PCR buffer. As positive control a PCR was performed with 0.5ul (2.5 units) commercial Taq. DNA polymerase (+)

Discussion

DNA polymerase from *T. aquaticus* has become a common reagent in molecular biology because of its utility in DNA amplification and DNA sequencing protocols [1, 6, 9, 10]. A simplified method is described here for cloning, expression and purification of recombinant *Taq* enzyme after overproduction in *E. coli*. Purification requires 1h heat-treating the *E. coli* lysate at 75°C, followed by centrifugation. The resulting enzyme contains a single, nearly homogeneous protein of the Taq DNA polymerase with a molecular size of 94 kDa as compared with commercial enzyme (Fig. 1). Under optimized conditions such as using a RD-buffer, 0.5µl of purified Taq yielded the same amount of PCR product as 2.5 units' according to 0.5µl of commercial enzyme (Fig. 2). The enhancing effect of RD-buffer is based on obtaining ingredients as ammonium sulfate and BSA, bind to and inactivate the putative inhibitors in the PCR [11-13].

The existence of inhibitory agents could be a consequence of sub optimized purification of enzyme [14-16] explaining the failure of PCR products with 0.5µl recombinant enzyme (Fig. 2). On the other site, 2µl of recombinant Taq polymerase yielded similar amount of PCR product compared to the reaction performed by 0.5µl commercial enzyme (Fig. 3). Grimm *et al.* [8] recently introduced a technique for enzyme purification known as "freezing and thawing method" that is based on rapid temperature change from -70 to -75°C, so that most of the host *E. coli* proteins could be denatured, and then were then easily removed from the lysate as a precipitate. This could be an alternative way to the boiling method as we have done to get more purified enzyme from cell lyses [17, 18].

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