Molecular Cloning and Characterization DNA Polymerase I from thermophilic Geobacillus SBS 4S strain

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Research article

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Abstract

Thermostable DNA polymerases are extensively used in biotechnology and life science applications. DNA Polymerase I was isolated from a hyperthermophile bacteria Geobacillus SBS 4S. Primers were designed using the template sequence of DNA Polymerase I gene of Geobacillus kaustophilus HTA26 strain. Nco 1 and Hind III sites were introduced on the forward and reverse primers respectively. Polymerase I gene of 2.6 Kb was cloned in pTZ57/ RT vector. Cloned gene of Polymerase I was restricted with Nco 1 and Bam H1, and ligated to pET 22b vector. Nco 1 site was used to insert twenty two N-terminal aminoacids (pelB) leader sequence at the start of the gene, which lead the recombinant protein in the periplasmic space, which increases the half life of recombinant protein. pelB fused with DNA Polymerase I produces soluble protein, which was detected after sonication. Sequencing shows that DNA polymerase I consists of 2499 bp with encodes for 832 amino acids, showed 99 % similarity with Geobacillus Kaustophillus . The expression of pelB fused DNA Polymerase I was optimized at different concentrations of IPTG and lactose. Highest expression was observed with 0.5mM IPTG and 20mM lactose. After Harvesting and sonication of BL21 codon plus cells, Polymerase I was produced in the soluble fraction. The supernatant containing the protein of interest, was separated after centrifugation at 10,000 rpm for 20 min. The protein was purified by ammonium sulphate precipitation and cation exchange column. The activity of purified DNA Polymerase I was checked by PCR reaction.

Background

DNA polymerases are key enzymes that are involved into DNA replication and repair processes that occur in all living organisms (1). Thermostable DNA polymerases are produced by thermophilic bacteria are valuable source of DNA polymerases. Thermophilic and hyperthermophilic enzymes have more importance over mesophilic enzymes, the most important application of thermostable enzyme is DNA polymerase. The most important application of DNA polymerase is in PCR. In prokaryotes there are three main types of DNA polymerases, Polymerase I, II and III. DNA polymerase III is the enzyme involved in DNA synthesis, whereas Pol I, and II has polymerase as well as exonuclease activity.

Synthesis of DNA by DNA polymerase occur in four steps. In the first step polymerase enzyme binds to the template primer dimer. In the second step dNTPS binds to enzyme template primer complex, in the third step phosphodiester bond is formed as a result of nucleophilic attack, In the final step a conformational change cause elimination of pyrophosphate moiety (2).

The first DNA polymerase was isolated by Kornberg and colleagues from E.coli in 1950s. This polymerase contains all three basic activities, 3’–5’ exonuclease, 5’–3’ exonuclease and polymerase activities. Up to today more than 100 DNA polymerases have been cloned and sequenced from many organisms including thermophile and archea. Most of the thermostable DNA polymerases have been isolated from Thermus genus especially from Thermus aquaticus. Taq polymerase is the first thermostable enzyme that has been characterized. DNA polymerases from thermophiles lack 3’–5’ exonuclease activity while others from hyperthermophiles retain all the three basic activities of E.coli.
polymerases(3). A few of thermostable DNA polymerases have also been characterized and purified from the thermophilic members of family Bacillaceae especially from genus Bacillus and new genus Geobacillus: B. stearothermophilus, B. caldoxylyoticus, B. caldotenax etc. DNA polymerase from Bacillus stearothermophilus is used in sequencing reactions (4).

Genus Geobacillus was proposed by Nazina et al Geobacillus showed strong similarity to B. kaustophilus, B. stearothermophilus, B. thermocatenulatus, B. thermodenitrificans, B. thermoglucosidasius, and B. thermoleovorans. species belonging to this genus have unique properties like rod shaped cells, aerobic or facultative aerobic cylindrical endospore formers, with optimum growth temperature at 37–75 °C. Bacteria belonging to genus Geobacillus shows optimum growth at 65°C and pH, 6.05–8.0 (5).

Methods

Samples of Geobacillus SBS 4S was taken from Prof. Dr. Naeem Rashid (School of Biological Sciences). Total DNA was isolated from bacterial cell, using classical method of Sambrook and Russels.(6) All the chemicals and kits. PCR amplification kit was from Thermofisher Scientific. For plasmid DNA extraction “Vivantis Nucleic Acid Extraction” kit was used. InsTAclone™ PCR cloning kit was of vivantis. EcoRI, HindIII and BamH1 was from Thermofisher Scientific. Thermo Scientific GeneJet™ MiniprepKit.

PCR amplification and primer designing

The sequence of DNA Polymerase I of Geobacillus kaustophilus was retrieved from NCBI database (GenBank: JYBP01000003.1). Primers were designed manually and checked on oligo nucleotide property calculator. Restriction sites were introduced at the start and end of the gene. One forward (Pol-F), one reverse primer (Pol-R) and one internal primer (Pol-I) were designed (Table 1).

PCR optimized with 0.15mM MgCl₂, 0.25 mM DNTPS, 100 pmoles of forward (Pol-F) and reverse primer (Pol-R) and 5U of Taq DNA polymerase (Fermentas). PCR was performed with initial denaturation at 94°C for 3 min, denaturation at 94 °C for 30sec, annealing at 55°C for 90 sec, extension at 72 °C for 1min and final extension at 72 °C for 10min. The PCR product was extracted by Vivantis Quick Gel Extraction kit. The PCR samples was visualized on agarose gel and “Vivantis Nucleic Acid Extraction” kit was used to extract PCR product from agarose gel.

Cloning and Transformation of DNA polymerase I in pTZ57R/T vector

After Gene clean purified gene (2.6 kB) sample was cloned into cloning vector using ligation kit (InsTAclone™ PCR cloning kit). PCR product (2.6kB) Vector pTZ57R/T were mixed with 10 X ligation buffer (3ul) and incubated at 22 °C for overnight in water bath. DH5α competent cells were transformed with ligated plasmids (Pol-pTZ57R/T5.4). 200 ul of competent cells and 15 ul of ligation mixture was incubated on ice for 40 min. then 90 sec heat shock was given to the sample and immediately placed sample on ice for 5–10min. After adding 800 ul of LB media into eppendorf sample mixture was placed
in shaker for 2 hr incubation. 200 ul of sample was taken and spread on 2 agar plates (1.5 % agar, 100 µg/ml of ampicillin, 1.33 µg/ml 5-bromo-4-chloro-3-indolyl-β-D-galactoside (X-gal) and 35 µg/ml of 0.4 M isopropylthio-β-D-galactoside (IPTG). Plates were placed in oven at 37 °C overnight. Pol-pTZ57R/T5.4 was isolated with Thermo Scientific GeneJet™ MiniprepKit.

The sequencing of Polymerase I gene was done with forward and reverse primers of M13 and internal primer (Pol–1) on Beckman Coulter (CEQTM 8000 DNA sequencer). Sequence was aligned with Clustal W and DDBJ (DNA Data bank Japan).

Cloning and Transformation of DNA polymerase I in pET 22b vector

Pol-pTZ57R/T vector was restricted with Ncol first, in 50 ul reaction mixture, with 25 U of Nco1 and 1 µg of Pol-pTZ57R/T vector and incubated at 37°C for 3hrs. The reaction mixture was kept at −20°C overnight. Next day 25 U of BamH1 was added and incubated at 37°C for 3 hr. Sample was visualized on the agarose gel. Polymerase I gene insert (2.6 kb) was ligated to pET22b vector in a 30 µl reaction with gene to vector ratio of 3:1 and 100 U of T4 DNA ligase and incubated at 22 °C overnight. Presence of insert (pol) into pET 22b vector was confirmed with double digestion with Ncol and BamHI.

Expression of Polymerase I in codon plus BL–21 cells

Pol-pET22b8.0 was transformed to BL–21 codon plus cells. Single transformed colony was inoculated in 10 ml LB ampicilin flask and incubated at 37 °C in shaker overnight. 1 ml culture was taken from inoculum and inoculated in 50 ml LB fresh media flask and incubated at 37°C in shaker until optical density (OD) 600 reached to 0.4–0.6. At this stage 0.05, 0.1, 0.2, 0.5 mM IPTG and 2, 5, 10 15 and 20 mM lactose was added to the culture and incubated for 5–6 hours. From this induced culture pellet was made in 50 ml falcon. Pellet was dissolved in 1.5 ml of 50mM tris-Cl pH 6.8. Equal amount of pellet mixture and 2X loading dye was mixed in an eppendorf and heat shocked for 3–5 min. sample was run on sodium dodecyl sulphate poly acrylamide gel electrophoresis (SDS-PAGE). The supernatant was checked for polymerase I.

Large scale preparation of Polymerase I

500ml of LB broth, inoculated with BL–21 codon plus cells (Pol-pET22b8.0) and induced with 5 mM lactose incubated at 37°C for 6 hr. The culture was 6500g for 15 min. Supernatant was discarded and 10 ml of 50mM tris-Cl pH 6.8 was added to the pellet. The procedure was repeated twice to wash the pellet.

Sonication to lye the cells

In 50 ml falcons the samples were sonicated for 1 hr. Samples were sonicated as 30 sec cycle and after each cycle 1 min rest was given, whole sonication procedure was carried by keeping samples on ice. Samples were centrifuged at 10,000g for 20 minutes and shifted the supernatant to sterile falcons and re-
suspended pellet in 10 ml 50mM Tris-Cl pH 6.8. Soluble fraction (supernatant) and insoluble fraction (pellet) was checked for polymerase I. Supernatant and pellet were analyzed on SDS-PAGE.

Expression of Polymerase I in codon plus BL–21 cells

Pol-pET22b8.0 was transformed to BL–21 codon plus cells. Single transformed colony was inoculated in 10 ml LB ampicilin flask and incubated at 37 °C in shaker overnight. 1 ml culture was taken from inoculum and inoculated in 50 ml LB fresh media flask and incubated at 37°C in shaker until optical density (OD) 600 reached to 0.4–0.6. At this stage 0.05, 0.1, 0.2, 2mM, 5mM, 10mM, 15mM and 20mM lactose concentration was added to the culture and again incubated culture in shaker for 5–6 hours. From this induced culture pellet was made in 50 ml falcon. Pellet was dissolved in 1.5 ml of 50 mM Tris-HCL buffer (pH 8). Equal amount of pellet mixture and 2X loading dye was mixed in an appendorf and heat shocked for 3–5 min. sample were loaded on sodium dodecyl sulphate poly acrylamide gel electrophoresis (SDS-PAGE). The supernatant was checked for polymerase I.

Purification of polymerase I

Supernatant was saturated with 30%, 40%, 50%, 60%, 70% & 80% ammonium sulphate. All the samples were analyzed on 12% SDS PAGE. The protein of interest was precipitated on 40% saturation with ammonium sulphate. The quantity of the protein in the sample was estimated by the UV absorption method. Pre packed Hitrap™ SP Sepharose Fast Flow 5 ml cation exchange column was used to carry out the ion exchange chromatography for purification of protein. The column is packed with sepharose which is 6% highly cross linked agarose and is a strong cation exchanger. Bead size of the resin is 45 - 165 µm, stable at pH 4 –12, stored at 4 to 30°C in 20% ethanol. The column was run at rate of 1 ml/min. after collecting the flow through and washings the salt gradient was applied from 0—1M NaCl and fractions were collected in sterile appendorfs by monitoring the peaks on chromatogram to analyze on SDS-PAGE.

Enzyme activity

PCR amplification of the polymerase I gene was done to check the activity of the enzyme (pol–1) by using Pol-F and Pol-R primers. Reaction mixture was prepared in three PCR tubes. Own heat treated crude enzyme extract was added in one tube, purified protein was added to second tube & commercial taq polymerase (Thermofisher) was added to the third tube. The conditions used for PCR were initial denaturation at 94°C for 3 minutes, denaturation at 94°C for 30 sec, annealing at 55°C for 1 minute, extension at 72°C for 1 minute and final extension at 72°C for 10 minutes. PCR product was analysed on 1% agarose gel.

Results And Discussion

Amplification of Polymerase I gene from Geobacillus kaustophilus
PCR was optimized by using template sequence of *Geobacillus kaustophilus* (figure 1) at 94°C for 3 min, denaturation at 94°C for 30 seconds, annealing at 55°C for 1:30 min, elongation at 72°C for 1 minute and final extension for 10 minutes at 72°C.

PCR product of 2.6 Kb (figure 2) was excised from agarose gel and ligated with pTZ57R/T and restricted with NcoI and BamHI and ligated to pET 22b. Cloning in pET22b was confirmed by Nco I and BamHI (figure 3 and figure 1s-supplementary file).

Expression of polymerase I was checked on BL21 codon plus cells, transformed with pol-pET22b8.0. Culture was induced with 0.05, 0.1, 0.2, 0.5 and 1mM IPTG and 2, 5,10, 15 and 20mM lactose. A band of 99kDa shows expression of polymerase I (Figure 4 and 5b).

Maximum expression of polymerase I was observed with 20mM lactose. After sonication the analysis of protein showed, polymerase I was present in supernatant of sonicated samples.

After harvesting cells in 50mM Tris buffer (pH 6.8), the cell suspension was lysed by sonication and the suspension was centrifuged to obtain the supernatant as the desired protein was soluble and it was present in the supernatant fraction. The protein was purified by heat treating the cell lysate at 60 °C for 20 min and centrifuging at 10,000 rpm for 20 min and separating the supernatant (figure 6). The heat treated lysate was further purified by passing through cation exchange column, desired protein was eluted at 1M NaCl concentration. Polymerase I was precipitated at 40% saturation of ammonium sulfate.

On FPLC column polymerase I was eluted with 1M concentration of NaCl, 5.86mg of protein was loaded on ion exchange column and 1.952 mg (figure 5a).

DNA polymerase I has 5’–3’ polymerase activity and 3’–5’ exonuclease activity. It fills the gaps which arises during DNA replication, repair and recombination. Sequencing results shows ten silent mutations in DNA polymerase I gene of *Geobacillus* SBS 4S (figure 2s).

*Polymerase I from Geobacillus sp.* was found to be 99KDa protein on SDS-PAGE, *Taq* DNA polymerase was cloned and expressed in *E.coli.* and molecular weight determination on SDS-PAGE showed a 94 KDa protein. The purified enzyme retained the activity comparable to commercial *Taq* DNA polymerase (7).

The gene coding for DNA polymerase I from *Geobacillus* Sp. SBS 4 strain was cloned in to the pET 22 b+ expression vector using Ncol and BamHI restriction sites. The construct was transformed in the *E.coli* strain BL 21 codon + and was grown on Luria-Bertani media (LB).

The Isopropyl-β-D-thiogalactopyranosid (IPTG) was used as an inducer of interested gene expression under the control of T7 promoter by using 0.05mM, 0.1mM, 0.2mM, 0.5mM, 1mM IPTG induction. The optimization of enzyme induction by IPTG with shake flask was found to be 0.5mM at exponential growth phase. The lactose was used as an inducer of expression of gene of interest under the control of T7 promoter by using 2mM, 5mM, 10mM, 15mM and 20mM lactose induction. The optimization of enzyme induction by lactose with shake flask was found to be 20mM at exponential growth phase. The
expression of gene of interest was very high with the lactose inducer and lactose is also a cheaper chemical as compared to IPTG which is an expensive chemical, so lactose inducer was used for further large scale culture preparation.

DNA polymerase I is a single polypeptide chain encoded by pol A gene. It consists of three distinct domains C, N and central domains. Domain C involves into main DNA polymerase activity while central domain and domain N are involved in 3’ – 5’ exsonuclease activity and 5’ – 3’ exonuclease activity respectively (8). Domain C is most important domain that is further divided into three subdomains designated as thumb, palm and fingers with highly conserved motifs A, B and C respectively (9). The palm subdomain has catalytic center and contains conserved carboxylate residues while subdomains fingers and thumb are involved into binding of DNA and incoming dNTPs during replication (10). Ten silent mutations were observed in polymerase I gene with one silent mutation in the C-terminal domain.

Cloning of DNA polymerase I from a thermophilic Rhodothermus marinus was done in E.coli. Sequence analysis of protein showed mutation at 756 position where phenylalanine was substituted by tyrosine. The protein polymerization activity was found to be 3100 units/mg of protein with retaining 3’ – 5’ and 5’ – 3’ exonuclease activities (11).

DNA polymerase from Pyrococcus KOD1 strain encode for 1671 amino acids polymerase I gene was expressed in E.coli (12). DNA polymerase from Geobacillus sp. SBS 4S was sequenced and purified for the first time. After heat treatment, ammonium sulphate precipitation and FPLC the protein was 99 % pure and was successfully used in a PCR reaction.

Conclusions: DNA polymerase I gene from a local isolate Geobacillus SBS 4S was successfully cloned and expressed in E.coli system. PeB leader sequence attached to the N-terminus of the protein makes the protein soluble. The expresson was optimized at 0.5mM concentration of IPTG and 20mM lactose. Polymerase I was purified by ammonium sulphate precipitation and ionexchange chromatography.

Declarations

Abbreviations: not applicable

Ethics approval and consent to participate: not applicable

Consent for publication: Not applicable

Availability of data and materials: Not applicable

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Authors’ contributions: Farheen Aslam and Saima Iftikhar Bajwa conceived and designed the experiments, analyzed the data and wrote the paper.
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### Tables

Table 1: Sequence, GC content, melting temperature and restriction sites of primers.

| Primer name | Sequence 5’-3’                                      | GC content (%) | Number of nucleotide | Melting temperature | Restriction sites |
|-------------|-----------------------------------------------------|----------------|----------------------|---------------------|-------------------|
| Pol-F       | CCATGGCCTTGAAAAAAAAGCTTGTTT                       | 37.04          | 27                   | 63.7                | CCATGG           |
| Pol-R       | AAGCTTTTATTTTCGTCATACCATGTCGAG                     | 42             | 31                   | 69.7                | AAGCTT           |
| Pol-I       | ATGACATCGTCTATCAAGGGGAAGACC                       | 48             | 27                   | 59.7                | -                 |

*Forward Primer contains NcoI Restriction site (CCATGG)*

**Reverse Primer contains HindIII Restriction site (AAGCTT)**
Figure 1

Sequence of Polymerase I gene (NCBI) and sequence of primers. Restriction sites shown in green. Start and stop codons are shown in pink.
Figure 2

Agarose gel electrophoresis showing PCR amplification of Polymerase I gene. Lane M: Gene Ruler DNA ladder mix. Lane 1 and 2: PCR product of 2.6kb.

Figure 3

Agarose gel of restriction analysis of pol-pET 22b 8.0 with NcoI and BamHI. M: DNA marker, lane 1: pol-pET 22b 8.0 restricted with NcoI and BamHI.

Figure 4

SDS PAGE of induced and uninduced sample of BL21 cells containing Pol-pET 22b 8.0. Lane 1, 2, 3, 4, and 5 showed polymerase I expression with 0.05, 0.1, 0.2, 0.5 and 1mM concentration of IPTG. Lane 6: uninduced sample.
Figure 5

(a) Purified Polymerase I after ion exchange chromatography on 12% SDS-PAGE. (b) Expression of Polymerase I with 2mM, 5mM, 10mM, 15mM and 20mM concentration of lactose.

Figure 6

The supernatant and pellet checked after heat treatment at 60°C. Lane 1: without heat treatment. Lane 2 & 3 supernatant and pellet after heat treatment at 60°C.

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