UDC 602.6; 604.6

MODIFICATION OF TAQ DNA POLYMERASE WITH IMPROVED ELONGATION ABILITY

Sarsen A.[1,2], Akishev Zh.[1], Saginova M.[1,2], Sultankulov B.[1], Khassenov B.[1]

[1]National Center for Biotechnology
13/5, Korgalzhyn road, Nur-Sultan, 010000, Kazakhstan
[2]L.N. Gumilyov Eurasian National University
Satpayev str., 2, Nur-Sultan, 010000, Kazakhstan
khassenov@biocenter.kz

ABSTRACT

Thermostable polymerases play a significant role in molecular biology and diagnostic practice. The most famous and demanded is Polymerase I from the thermophilic bacterium *Thermus aquaticus* (Taq-pol). This polymerase at one time made a kind of revolution in the polymerase chain reaction. In this work, we attempted to modify this polymerase by attaching an additional Sso7d protein from *Sulfolobus solfataricus* to Taq-pol, which provides additional binding to the double-stranded DNA of the template. *Sso7d-Taq* fusion gene was expressed in BL21(DE3) cells. Optimal conditions were selected for maximum production of modified Sso7d-Taq polymerase. The optimal conditions for the intracellular accumulation of *Sso7d-Taq* polymerase: activation of the T7 promoter when the optical density of the culture reaches OD_{600} = 0.8-1.0 by adding IPTG at a concentration of 0.2 mM, followed by incubation of the culture at 37°C for 20-24 hours. Recombinant *Sso7d-Taq* polymerase has been purified and tested by PCR for thermal stability and elongation time. It was found that the *Sso7d-Taq* enzyme withstands 5 hour incubation at 95°C and 75 minute incubation at 98°C. Comparative analysis with unmodified Taq DNA polymerase showed that the *Sso7d-Taq* enzyme reduces the elongation rate by several times - up to 15-13 seconds per 1 kbp. The results obtained indicate the prospects of using *Sso7d-Taq* DNA polymerase in scientific research and diagnostic practice.

Key words: DNA polymerase, PCR, *Thermus aquaticus*, Sso7d-Taq-pol, thermostability.

INTRODUCTION

DNA dependent polymerases are essential for all living organisms to survive and reproduce. In most cases, cells contain DNA polymerases belonging to different classes and designed to solve different problems. The dominant role is played by DNA polymerases involved in genome replication during the cell cycle. These polymerases must be highly processive [1]. Other non-replicative polymerases are involved in filling in the gaps in the DNA structure resulting from DNA damage or DNA repair. They are more fidelity, but not highly processive [2]. Non-replicative DNA polymerases are widely used in scientific research, sequencing and diagnostic practice [3, 4]. The known

and practically used DNA polymerase is Polymerase I (PolI) from the thermophilic bacterium *Thermus aquaticus*, also called Taq DNA polymerase [5]. Taq DNA polymerase is used in molecular biology and molecular diagnostic systems as an enzyme in the polymerase chain reaction (PCR) [6]. High polymerase properties of Taq DNA polymerase stimulates studies of this enzyme in order to improve its properties [7-9].

The aim of this work was to modify Taq DNA polymerase to decrease elongation time in PCR. We believe that this will improve the characteristics of polymerase and will be of great practical importance in molecular genetic research and diagnostic practice.

Materials and methods

Vectors, strains, enzymes and chemicals. Plasmid *pET-28c(+)* (Novagen) was used to construct the expression vector. The gene of Taq Polymerase of *Thermus aquaticus* (Taq-pol, 2496 bp) and gene Sso7d of *Sulfolobus solfataricus* (Sso7d, 189 bp) were used from NCB lab collection. Polymerases Pfu (Thermo Fisher, USA) and Taq (NCB, Kazakhstan) and restriction enzymes NdeI, EcoRI (Thermo Fisher, USA) were employed for the amplification and cloning of the target genes. *Escherichia coli* strain DH5α was acquired from Thermo Fisher Scientific. BL21(DE3) cells were purchased from Invitrogen (USA). The chemical reagents used in this study were of molecular biology or pure analytical grade and purchased from Sigma-Aldrich (St. Louis, USA) and AppliChem (Darmstadt, Germany). The vectors and enzyme were stored at -20°C, the strains were stored at -80°C and chemicals were stored accordingly manufactured recommendations. The table 1 shows the sequences of the oligonucleotides used in the work.

Table 1. Oligonucleotides

Oligonucleotides	The nucleotide sequence
Sso7dfw	5’-GCAATTCCATATGACCGTAAAGTTCAAGTACAAA-3’
Sso7drv	5’-CGGAATTCGCCGGTACCCTTTTTCTGCTTCTCCAGCAT3’
TaqEcoRIfw	5’CGGAATTCGGTGGCGGTGCAATGAGGGGGATGCTGCCCCT-3’
TaqEcoRIrv	5’-CGGAATTCTCACTCCTTGGCGGAGAGCCA-3’
TaqPol_706R	5'-CCCCGGGAAGGTTGTCGGACTCGTC-3'
T7fw	5`-TAATACGACTCACTATAGGG-3`
T7rv	5'-GCTAGTTATTGCTCAGCGG-3'
AmyBLfw	5’-ATGAAACAACAAAAACGGCTTTAC-3’
AmyBLrv	5’-TCTTTGAACATAAATTGAAACCGA-3’
18S1F	5'-TACCTGGTTGATCCTGCCAGTAG-3'
18S1R	5'-TAATATACGCTATTGGAGCTGG-3'

Cloning of the gene and vector construction. The cloning was done by two stages. The first stage – cloning the *sso7d* gene into pET-28c(+). The *sso7d* gene was amplified with PCR primers Sso7dfw and Sso7drv. PCRs (50 μL final volume) contained 5 μL of 10X Pfu Buffer (Thermo Fisher), 3 μL of 25 mM $MgCl_2$, 5 μL of

dNTPs (a 2 mM stock solution), 1 μL of each primer (a 10 μM stock solution), 100 ng of a DNA template, 2 μL of Pfu polymerase (1250 U/mL), and 33 μL of nuclease-free water. The following amplification parameters were utilized: initial denaturation at 98°C for 1 min; then 30 cycles of 98°C for 30 sec, 50°C for 45 sec, and 68°C for 30 sec; and final extension at 68°C for 10 min. The PCR-product was purified by phenol/chloroform extraction and was cloned into the pET-28c (+) vector on cites NdeI/EcoRI. PCR-product and pET-28c(+) were hydrolyzed NdeI and EcoRI endonucleases in O Buffer (Thermo Fisher, USA). The T4 DNA Ligase (NEB, UK) was used for ligation. The competent cells of DH5α were treatment with ligation mixture by heat-shock protocol. The clones were screened by PCR with T7 primers. The DNA were extracted by using of MiniPrep Kit (Thermo Fisher, USA) and sequenced on T7 region. The second stage – cloning *taq-pol* gene into the resulted vector pET-28c (+)/Sso7d. The *taq-pol* gene was amplified with PCR primers TaqEcoRIfw and TaqEcoRIrv. PCRs (50 μL final volume) contained 5 μL of 10X Pfu Buffer (Thermo Fisher), 3 μL of 25 mM $MgCl_2$, 5 μL of dNTPs (a 2 mM stock solution), 1 μL of each primer (a 10 μM stock solution), 100 ng of a DNA template, 2 μL of Pfu polymerase (1250 U/mL), and 33 μL of nuclease-free water. The following amplification parameters were utilized: initial denaturation at 98°C for 1 min; then 30 cycles of 98°C for 30 sec, 58°C for 45 sec, and 68°C for 3.5 min; and final extension at 68°C for 10 min. The PCR-product was purified by phenol/chloroform extraction and was cloned into the *pET-28c(+)* vector on EcoRI cite. PCR-product and pET-28/Sso7d vector were hydrolyzed EcoRI endonuclease in EcoRI Buffer (Thermo Fisher, USA). The T4 DNA Ligase (NEB, UK) was used for ligation. The competent cells of DH5α was treatment with ligation mixture by heat-shock protocol. The clones were screened by PCR with Sso7dfw and TaqPol_706R primers. The plasmid DNA were extracted by using of MiniPrep Kit (Thermo Fisher, USA) and sequenced on T7 region.

Sequencing. Sequencing of inserted gene was carried out on an ABI 3730xl Genetic Analyzer (Applied Biosystems) using BigDye Terminator v3.1 (Applied Biosystems).

Producing strain preparation. The competent cells of BL21(DE3) strain were electroporated (MicroPulser BioRad, USA). The transformed cells were selected on LB-agar with kanamycin (50 μg/mL). Colonies were picked and grown in 10 mL LB-broth with kanamycin at 37°C and 180 rpm for 8 h. Cells harvested by centrifuging at 6000 × g, 4°C, 7 min. Supernatant was discarded and pellet was suspended in 3 mL of sterile LB-broth with 50% glycerol and aliquoted. Tubes with producing strain were stored at -80°C.

Cell density optimization for Sso7d-Taq-pol production. Cells from -80°C were inoculated in the three tubes with 5 mL of LB-broth with kanamycin and grow for 3 h at 37°C with shacking at 150 rpm. Each culture was transferred in the 500 mL flask with 100 mL LB-broth with kanamycin and grow at the same conditions to the cell density (OD_{600}) 0.6, 0.8 and 1.0, respectively. The recombinant cultures were inducted by IPTG in 0.5 mM. The inducted cultures incubated at the same conditions for overnight. Inducted culture was used for crude extract preparation and polymerase activity testing.

Temperature optimization for Sso7d-Taq-pol production. Cells from -80°C were inoculated in the two tubes with 5 mL of LB-broth with kanamycin and grow for 3 h at 37°C with shacking at 150 rpm. Each culture was transferred in the 500 mL flask with 100 mL LB-broth with kanamycin and grow by shacking (150 rpm) at 30°C and 37°C, respectively to the optimal cell density. The recombinant cultures were inducted

by IPTG in 0.5 mM. The inducted cultures incubated at the same temperatures for overnight. Inducted culture was used for crude extract preparation and polymerase activity testing.

Optimization of IPTG concentration for Sso7d-Taq-pol production. Cells from -80°C were inoculated in the three tubes with 5 mL of LB-broth with kanamycin and grow for 3 h at 37°C with shacking at 150 rpm. Each culture was transferred in the 500 mL flask with 100 mL LB-broth with kanamycin and grows by shacking (150 rpm) at optimal temperature to the optimal cell density. The recombinant cultures were inducted by IPTG in 0.2, 0.5 1.0 mM, respectively. The inducted cultures incubated at the same conditions for overnight. Inducted culture was used for crude extract preparation and polymerase activity testing.

Optimization of time induction for Sso7d-Taq-pol production. Cells from -80°C were inoculated in the tube with 5 mL of LB-broth with kanamycin and grow for 3 h at 37°C with shacking at 150 rpm. The culture was transferred in the 2000 mL flask with 300 mL LB-broth with kanamycin and grow and by shacking (150 rpm) at optimal temperature to the optimal cell density. The culture was induced by IPTG in optimal concentration. The inducted cultures incubated for 24 h. After 16 h and 20 h 100 mL culture was collected and used for crude extract preparation and polymerase activity testing.

Crude enzyme preparation. 100 mL of culture was centrifuged (6000 × g, 7 min, 4°C). Supernatant was discarded and pellet was suspended in 1 ml of 20 мM Tis-HCl (pH 7.4), 20 mM NaCl and sonicated by Omni Sonic Ruptor-4000 (USA) for 10 min. The lysate was clarified by centrifugation (15800 × g, 30 min, 4°C). The supernatant was incubated at 70°C for 30 min and again clarified by centrifugation (15800 × g, 30 min, 4°C). Supernatant was stored at 4°C and used for PCR like enzyme.

Testing for polymerase activity of crude enzyme. For testing of crude enzyme used PCR. The PCR mix was contained: 2.5 µL of 10X Taq DNA Buffer (500 mM KCl, 0.8% (v/v) NP-40, 100 mM Tris-HCl, pH 8.8), 2 µL of dNTP mix (2.5 mM stock solution), 1.5 µL of 25 mM $MgCl_2$, 2 µL DNA (pET-28c(+) in concentration 30 ng/µL), 1µL of 10 µM forward primer (T7fw) 1µL 10 µM reverse primer (T7rv), 3 µL of crude enzyme and 12µL of MiliQ. PCR Program was: initial denaturation at 95°C for 3 min; then 30 cycles of 95°C for 30 sec, 55°C for 45 sec, and 72°C for 30 sec; and final extension at 72°C for 10 min.

Purification of Sso7d-Taq-pol. Cells from -80°C were inoculated culture was transferred in the 500 mL flask with 100 mL LB-broth with kanamycin and grow by shacking for overnight. Night culture was transferred to 450 fresh LB-broth with kanamycin and grows and inducted by IPTG. The cells were harvested by centrifugation (6000 × g, 4°C, 7 min). Pellet was suspended in 8 mL of Buffer S (20 mM Tris-HCl pH 8.0, 25 mM D-glucose, 1 mM EDTA, 20 mM KCl, 0.25% Tween-20, 0.25% Triton X-100) and lysed by 16 mg lysozyme (AppliChem, Germany) at room temperature for 30 min. The lysate was sonicated for 20 min. The lysate was clarified by centrifugation (18000 × g, 4°C, 20 min). Supernatant was incubated at 70°C for 1 h and again clarified by centrifugation (40000 × g, 4°C, 1 h). The supernatant was loaded into HiTrap Chelating 1 ml column (GE, USA) activated with $NiCl_2$ and equilibrated with Buffer A (20 mM Tris-HCl (pH 8.0), 20 mM Imidazole, 500 mM NaCl). The recombinant Sso7d-Taq-pol was eluted by linear gradient between Buffers A and B (20 mM Tris-HCl (pH

8.0), 500 mM Imidazole, 500 mM NaCl). The FPLC AKTA Purifier 10 (GE, USA) was used for protein purification. The fractions were analyzed by SDS-PAGE.

SDS-PAGE. Sodium dodecyl sulphate–polyacrylamide gel electrophoresis for electrophoretic separation of proteins was performed by in a 12% polyacrylamide gel according to the Laemmli method [10] in a miniProtean-IV cell (Bio-Rad Laboratories Inc). Electrophoresis conditions were as follows: 120 V for 90 min. The gel was stained in 2% Coomassie Brilliant Blue R250 and destained with 7.5% acetic acid in 25% ethanol. The molecular weight marker of proteins: 116, 66.2, 45, 35, 25, 18.4, 14.4 kDa (#26610, Thermo scientific) was used.

Investigation of thermostability of purified Sso7d-Taq-pol in PCR. Purified Sso7d-Taq-pol was incubated at 95 ° C and 98 ° C for 10 hours and 90 minutes, respectively. Next, PCR was carried out. The PCR mix was contained: 2.5 μL of 10X Taq DNA Buffer, 2 μL of dNTP mix (2 mM stock solution), 1.5 μL of 25 mM $MgCl_2$, 2 μL DNA (*pET-28c(+)* in concentration 30 ng/μL), 1μL of 10 μM forward primer (T7fw) 1μL 10 μM reverse primer (T7rv), 3 μL of *Sso7d-Taq-pol* and 12 μL of MiliQ. PCR Program was: initial denaturation at 95°C for 3 min; then 30 cycles of 95°C for 30 sec, 55°C for 45 sec, and 72°C for 30 sec; and final extension at 72°C for 10 min.

Investigation of time of elongation for purified Sso7d-Taq-pol in PCR. The elongation time in PCR changed by 1 kbp from 1 minute to 10 sec. The PCR mix was contained: 2.5 μL of 10X Taq DNA Buffer, 2 μL of dNTP mix (2 mM stock solution), 1.5 μL of 25 mM $MgCl_2$, 2 μL DNA (*pET-28c/AmyL* in concentration 30 ng/μL), 1μL of 10 μM forward primer (T7fw) 1μL 10 μM reverse primer (T7rv), 3 μL of *Sso7d-Taq-pol* and 12 μL of MiliQ. PCR Program was: initial denaturation at 95°C for 3 min; then 30 cycles of 95°C for 30 sec, 55°C for 1min, and 72°C for 1 min-10 sec; and final extension at 72°C for 10 min. Recombinant Taq DNA polymerase (NCB, Kazakhstan) was used as a reference polymerase (control).

PCR on genomic DNA with purified Sso7d-Taq-pol. The PCR on genomic DNA of *Bacillus licheniformis*, *Pichia pastoris* and *Bos taurus* was carried out. The primers AmyBLfw/AmyBLrv were used for amplification from *B.licheniformis* DNA and 18S1F/18S1R were used for amplification from *P.pastoris* and *B.taurus* DNA. The PCR mix was contained: 2.5 μL of 10X Taq DNA Buffer, 2.5 μL of dNTP mix (2 mM stock solution), 1.5 μL of 25 mM $MgCl_2$, 1 μL DNA (100 ng/μL), 1μL of 10 μM forward primer, 1μL 10 μM reverse primer, 3 μL of Sso7d-Taq-pol and 12.5 μL of MiliQ. PCR Program was: initial denaturation at 95°C for 10 min; then 30 cycles of 95°C for 30 sec, 55°C for 1 min, and 72°C for 1 min-15 sec; and final extension at 72°C for 10 min.

RESULTS AND DISCUSSUON

pET-28c/Sso7d-Taq construction. Taq polymerase consists of two structural and functional domains: a domain providing exonuclease activity in the 5'→3 'direction and a domain providing nucleotide attachment to the growing chain, also in the 5'→3' direction. Removal of the exonuclease domain is known to significantly reduce the processivity of Taq polymerase [11, 12]. It means that the exonuclease domain plays a significant role in the binding of Taq polymerase to DNA. Study of the structure of the Taq-DNA complex indicates a strong interaction between the exonuclease domain and template DNA [13]. A decrease in this interaction in the truncated *Taq* polymerase leads to a decrease in processivity [8]. The interaction can be enhanced by attaching an additional domain that ensures the stability of the Taq-DNA complex. The *Sso7d*

protein from *Sulpholobus solfactaricus* is a promising candidate for this. This protein binds to double-stranded DNA as a monomer [14-16]. It is possible to obtain modified Taq DNA polymerase with an added Sso7d domain using genetic engineering. Figure 1 shows the structure of this modified polymerase.

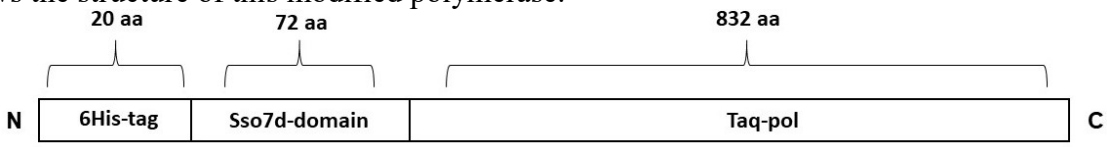


Fig 1. Structure of Sso7d-Taq polymerase

As can be seen from Figure 1, a hexahistidine tag is added to the Sso7d-Taq polymerase from the N-terminus to facilitate the purification procedure on divalent metal ions. The choice of vector pET-28c (+) as an expression vector was chosen for the following reasons: a strong and inducible T7 promoter, an operator region for binding of the LacI repressor protein, good copy number in *E. coli* cells, a convenient and rich polylinker with cloning sites, reliable antibiotic marker - kanamycin. The *sso7d* gene was chosen first for cloning, since the *taq-pol* gene contains many restriction sites, including NdeI. Figure 2 shows the result of PCR screening of clones after cloning of the *sso7d* gene in pET-28c (+).

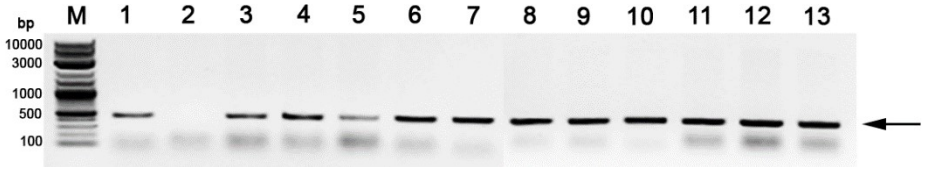


Fig. 2. PCR screening of *sso7d* clones

As can be seen from Figure 2, 12 out of 13 clones were positive. In contrast, as a result of cloning the *Taq-pol* gene in the pET-28/Sso7d vector, out of 10 tested by PCR, only one clone was successful (Fig. 3).

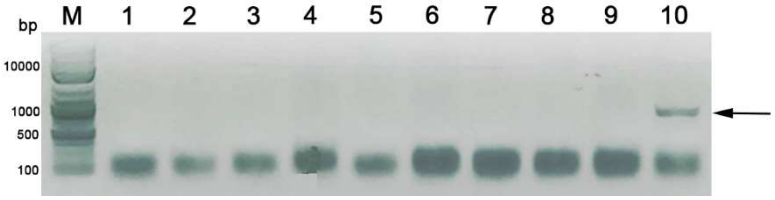


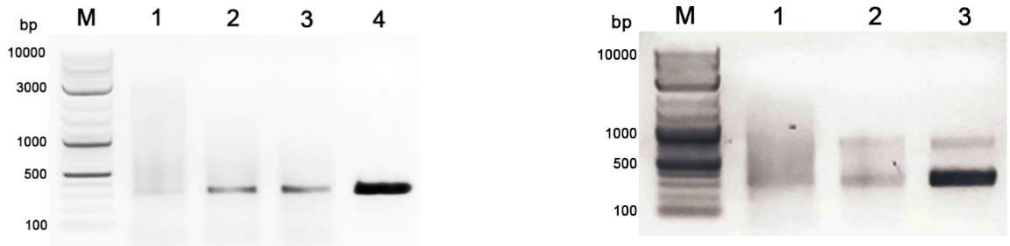
Fig. 3. PCR screening of *taq-pol* clones

Two factors contributed to the low efficiency of cloning: the large length of the Taq-pol gene (2496 bp) and the fact that the Taq-pol gene was cloned at one restriction site and both integration of the Taq-pol gene in the right direction and in the opposite direction was equally probable. But, nevertheless, the result is an expression vector carrying two genes linked in a covalent way: *Sso7d* and *Taq-pol*. Sequencing confirmed the absence of any mutations in the insert sequence. The full length of the protein in the open reading frame was 924 amino acid residues with a calculated molecular weight of 104 kDa. The sequence of Sso7d-Taq is:
MGSSHHHHHHSSGLVPRGSHMTVKFKYKGEEKEVDISKIKKVVWRVGKMISFTY
DEGGGKTGRGAVSEKDAPKELLQMLEKQKKGTGEFGGGAMRGMLPLFEPKG
RVLLVDGHHLAYRTFHALKGLTTSRGEPVQAVYGFAKSLLKALKEDGDAVIV
VFDAKAPSFRHEAYGGYKAGRAPTPEDFPRQLALIKELVDLLGLARLEVPGYE
ADDVLASLAKKAEKEGYEVRILTADKDLYQLLSDRIHVLHPEGYLITPAWLWE
KYGLRPDQWADYRALTGDESDNLPGVKGIGEKTARKLLEEWGSLEALLKNLD
RLKPAIREKILAHMDDLKLSWDLAKVRTDLPLEVDFAKRREPDRERLRAFLERL

EFGSLLHEFGLLESPKALEEAPWPPPEGAFVGFVLSRKEPMWADLLALAAARG
GRVHRAPEPYKALRDLKEARGLLAKDLSVLALREGLGLPPGDDPMLLAYLLDP
SNTTPEGVARRYGGEWTEEAGERAALSERLFANLWGRLEGEERLLWLYREVE
RPLSAVLAHMEATGVRLDVAYLRALSLEVAEEIARLEAEVFRLAGHPFNLNSR
DQLERVLFDELGLPAIGKTEKTGKRSTSAAVLEALREAHPIVEKILQYRELTKLK
STYIDPLPDLIHPRTGRLHTRFNQTATATGRLSSSDPNLQNIPVRTPLGQRIRRAFI
AEEGWLLVALDYSQIELRVLAHLSGDENLIRVFQEGRDIHTETASWMFGVPRE
AVDPLMRRAAKTINFGVLYGMSAHRLSQELAIPYEEAQAFIERYFQSFPKVRA
WIEKTLEEGRRRGYVETLFGRRRYVPDLEARVKSVREAAERMAFNMPVQGTA
ADLMKLAMVKLFPRLEEMGARMLLQVHDELVLEAPKERAEAVARLAKEVME
GVYPLAVPLEVEVGIGEDWLSAKE.

The producer strain obtaining and optimization of cultivation conditions. For the expression of *sso7d-Taq* in E. coli, the BL21 (DE3) strain was chosen, which is characterized by rapid growth on Luria-Bertani medium and a high level of expression of heterologous proteins [17, 18]. For maximum production of the recombinant Sso7d-Taq protein, the following conditions were optimized: the density of the bacterial culture at which the T7 promoter was activated, the induction temperature, the concentration of the IPTG induction activator, and the incubation time of the recombinant culture with IPTG. An important issue in the intracellular expression of a recombinant protein in E. coli is its detection among *E. coli's* own proteins. In most cases, the level of expression can be determined by comparing protein extracts of the culture before and after induction in SDS-PAGE. But at a low level of expression, this method is not applicable and more sensitive and specific methods are used: mass spectrometric analysis [19] or Western blotting using either protein-specific antibodies or antibodies to the protein label [20]. A completely different detection method is the enzymatic test. In this case, it is not the protein itself that is detected, but its enzymatic activity. In our studies, when optimizing the cultivation conditions and induction of the recombinant protein Sso7d-Taq, the polymerase activity of crude enzymatic extract for the samples were investigated and, based on a comparative analysis of the signal intensity for the PCR products in agarose gel, the optimal conditions were selected.

It was found that the optimal density of the culture BL21(DE3)-pET-28/Sso7d-Taq for induction is OD600 = 0.8-1.0 (Fig. 4A). At these values, the polymerase activity of the crude enzyme is higher than at OD600 = 0.6.

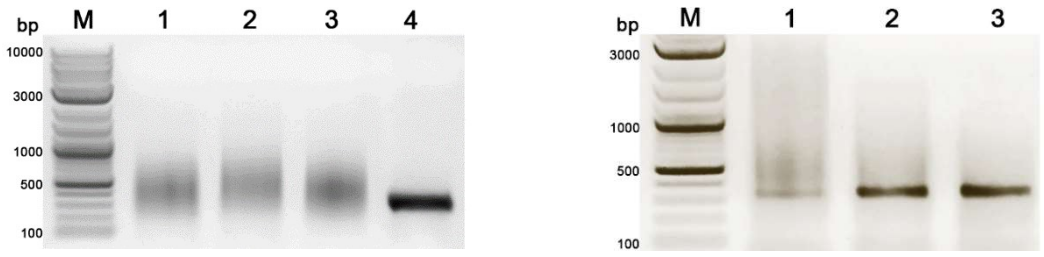


A — M-DNA ladder; 1-OD_{600}=0.6; 2-OD_{600}=0.8; 3-OD_{600}=1.0; 4-control

B — M-DNA ladder; 1-30°C; 2-37°C; 3-control

Fig.4. Effect of culture density (A) and induction temperature (B) on Sso7d-Taq expression in BL21(DE3)

More convenient induction temperature is 37°C than 30°C (Fig. 4B). The concentration of the IPTG, activating the T7 promoter, was investigated. Significant difference between PCR test results in samples between 0.2 mM, 0.5 mM, 1.0 mM was not observed (Fig. 5A).



A

M-DNA ladder; 1-0.2 mM; 2-0.5 mM; 3-1.0 mM; 4-control

B

M-DNA ladder; 1-16h; 2-20h; 3-24h

Fig. 5. Effect of IPTG concentration (A) and induction time (B) on Sso7d-Taq expression in BL21(DE3)

Optimization of the induction conditions over time showed that at least 16 hours is sufficient for the accumulation of Sso7d-Taq in BL21(DE3) cells (Fig. 5B), but a longer induction up to 20-24 hours increases the accumulation of the recombinant protein. Thus, the optimal conditions for the accumulation of Sso7d-Taq in the BL21(DE3) cells are: activation of the T7 promoter when the optical density of the culture reaches OD_{600} = 0.8-1.0 by adding IPTG at a concentration of 0.2 mM, followed by incubation of the culture at 37°C for 20-24 hours.

Isolation and purification of Sso7d-Taq. From experiments on optimization of cultivation conditions, it was known that the recombinant protein Sso7d-Taq is present in the BL21(DE3) lysate in a water-soluble fraction. Isolation using thermal heating in combination with high-speed centrifugation made it possible to significantly enrich the protein extract with Sso7d-Taq protein. Affinity chromatography on Ni^{2+} ions carried out in a linear gradient of imidazole (20-500 mM) allowed for high-quality protein purification (Fig. 6).

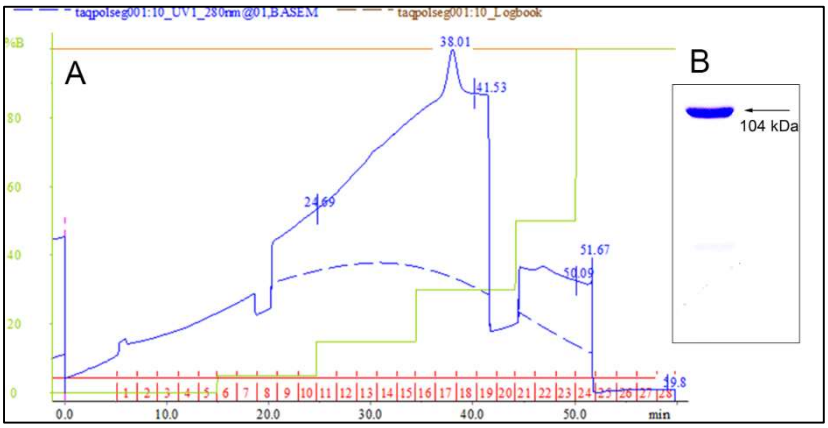
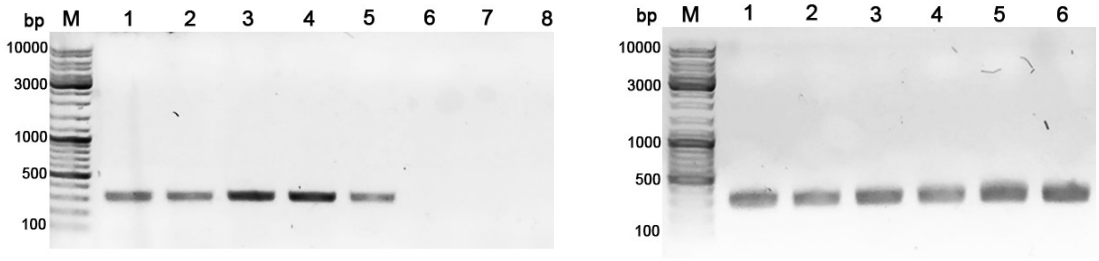


Fig. 6. Chromatography for purification recombinant Sso7d-Taq (A) and electrophoresis of fraction after purification Sso7d-Taq (B)

The chromatogram (Fig. 6A) shows that at an imidazole concentration of 150 mM, a peak is observed for the fraction, indicating the presence of protein in this fraction. Fraction electrophoresis showed that fractions 9-18 contain a protein corresponding to the molecular weight of Sso7d-Taq - 104 kDa (Fig. 6B). Fractions 9-18 were pooled and suspended in 50% glycerol and was used to test for polymerase properties of the recombinant Sso7d-Taq.

Enzymatic characteristics of Sso7d-Taq. A feature of the polymerase I from *Thermus aquaticus* is its thermal stability [11, 21, 22]. The thermal stability of the Taq-pol protein, both native and recombinant, has provided the popularity of this enzyme in molecular genetic studies [23]. Previously, we tested the thermal stability of two recombinant Taq-pol enzymes from *Thermus aquaticus* [24] and Pfu-pol from *Phyrococcus furiosus* [25]. Therefore, the question of the temperature stability of the obtained modified Sso7d-Taq enzyme is relevant. Incubation of the purified *Sso7d-Taq* protein at 95°C showed that the protein retains polymerase activity when incubated for up to 5 hours (Fig. 7A), which is a good indicator, although 1 hour less than the unmodified Taq-pol enzyme. [24].



A

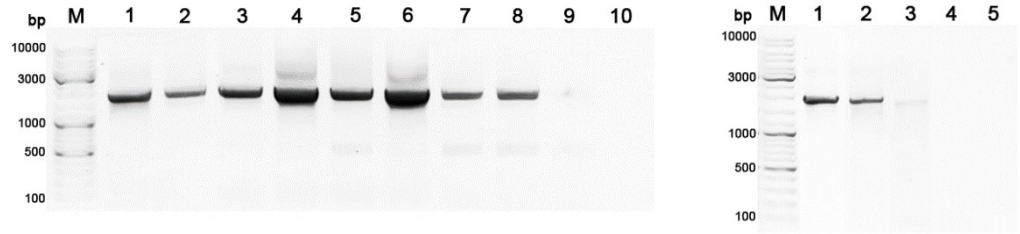
M-DNA Ladder; 1-0h, 2-1h, 3-3h, 4-4h, 5-5h, 6-6h, 7-8h, 8-9h, 9-10h

B

M-DNA Ladder; 1-0 min, 2-15 min, 3-30 min, 4-45 min, 5-60 min, 6-75 min, 7-90 min

Fig. 7. Effect of time incubation at 95°C (A) and 98°C (B) of purified Sso7d-Taq on its polymerase activity

At the same time, Sso7d-Taq retains polymerase activity when incubated at 98°C for 75 minutes (Fig. 7B), which indicates sufficient thermal stability of the Sso7d-Taq enzyme. It was noted that the presence of the Sso7d domain by 4.5 times increases the processivity of Taq-pol up to 104 nucleotides [8]. It seems interesting how the presence of Sso7d will reduce the elongation time in PCR. Figure 8A shows the results of experimental studies on amplification of the *amyL* gene with a length of 1700 bp.



A

M-DNA Ladder; 1-60 sec, 2-45 sec, 3-30 sec, 4-25 sec, 5-20 sec, 6-15 sec, 7-14 sec, 8-13 sec, 9-12 sec, 10-10 sec

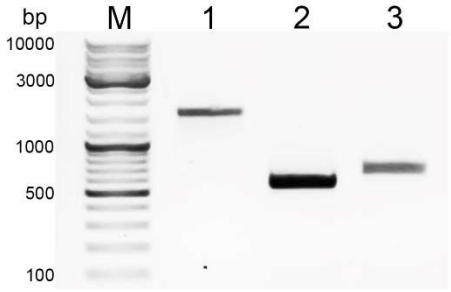
B

M-DNA Ladder; 1-60 sec, 2-45 sec, 3-30 sec, 4-25 sec, 5-20 sec

Fig. 8. Effect of elongation time Sso7d-Taq (A) and Taq polymerase (B) in PCR

In this series of experiments, the elongation time was reduced from 1 minute by 1 kbp to 10 seconds. As can be seen from the results, PCR takes place with a decrease in the elongation time up to 12 seconds by 1 kbp. Although it should be noted that the signal remains clear at 15-13 seconds at 1 kbp. At the same time, unmodified Taq DNA polymerase amplifies at 60-45 seconds and at 30 seconds the signal is very weak (Fig. 8B).

To test the polymerase activity of the Sso7d-Taq enzyme on genomic DNA, PCR was carried out on DNA samples from the bacterium (*Bacillus licheniformis*), yeast chromosomal DNA (*Pichia pastoris*) and calf thymus DNA (*Bos* taurus). The elongation time was 15 seconds per kbp. Figure 9 shows the results.



M-DNA Ladder; 1- *Bacillus licheniformis*, 2- *Pichia pastoris*, 3- *Bos taurus*
Fig. 9. Results of PCR for bacteria, yeast and animal genomic DNA

PCR using Sso7d-Taq was successful for the samples, which indicates the suitability of Sso7d-Taq DNA polymerase for amplifying loci with genomic DNA of prokaryotic and eukaryotic organisms.

CONCLUSION

Thus, by cloning two genes: *sso7d* from *Sulpholobus solfactaricus* and the full-length *polI* gene from *Thermus aquaticus*, a genetically engineered construct was created as part of the pET-28c (+) vector, which makes it possible to express the Sso7d-Taq fusion protein gene. This construct was used to transform competent cells of the *Escherichia coli* strain BL21 (DE3) and obtain a producing strain of the recombinant polymerase Sso7d-Taq. The optimal conditions for the intracellular accumulation of Sso7d-Taq: activation of the T7 promoter when the optical density of the culture reaches OD_{600} = 0.8-1.0 by adding IPTG at a concentration of 0.2 mM, followed by incubation of the culture at 37°C for 20-24 hours. Recombinant Sso7d-Taq polymerase has been purified and tested by PCR for thermal stability and elongation time. It was found that the Sso7d-Taq enzyme withstands a 5 hour incubation at 95°C and a 75 minute incubation at 98°C. Comparative analysis with unmodified Taq DNA polymerase showed that the Sso7d-Taq enzyme reduces the elongation rate by several times - up to 15-13 seconds per 1 kbp. The results obtained indicate the prospects of using Sso7d-Taq DNA polymerase in scientific research and diagnostic practice. Work in this direction will continue, in particular, to optimize conditions for increasing the efficiency of PCR.

Acknowledgement

This work was supported by the Ministry of Education and Science of the Republic of Kazakhstan, Science-Technical Program OR11465422 «Creation of a biobank of rare and endangered species of flora and fauna of Kazakhstan for the conservation of biodiversity» for 2021-2022.

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ЭЛОНГАЦИЯЛАУ ҚАБІЛЕТІ ЖЕТІЛДІРІЛГЕН TAQ ДНҚ ПОЛИМЕРАЗАСЫНЫҢ МОДИФИКАЦИЯСЫ

Сәрсен А.[1,2], Акишев Ж.[1], Сагинова М.[1,2], Султанкулов Б.[1], Хасенов Б.[1]

[1]Ұлттық биотехнология орталығы
Қорғалжын тас жолы, 13/5, Нұр-Сұлтан қ., 010000, Қазақстан
[2]Л.Н.Гумилев атындағы Еуразия Ұлттық Университеті
Сәтпаев көшесі, 2, Нұр-Сұлтан қ., 010000, Қазақстан
khassenov@biocenter.kz

ТҮЙІН

Термостабильді полимеразалар молекулалық биология мен диагностикалық практикада маңызды рөл атқарады. Ең танымал және сұранысқа ие - термофильді бактериядан алынған полимераза I *Thermus aquaticus* (Taq-pol). Бұл полимераза бір кездері полимеразды тізбекті

реакцияда өзіндік революция жасады. Бұл жұмыста біз *Sulfolobus solfataricus*-тен *Sso7d* қосымша ақуызын Taq-pol-ге қосып, полимеразды өзгертуге тырыстық, ол шаблонның қостізбекті ДНҚ-мен қосымша байланыстыруды қамтамасыз етеді. Sso7d-Taq гені BL21(DE3) жасушаларында көрсетілген. Модификацияланған Sso7d-Taq полимеразасының максималды өндірісі үшін оңтайлы жағдайлар таңдалды. Sso7d-Taq жасушаішілік жинақтау үшін оңтайлы жағдайлар: 0,2 мм концентрациясында IPTG қосу жолымен OD600 = 0,8-1,0 культураның оптикалық тығыздығына жеткен кезде T7 промоторын іске қосу, кейіннен 20-24 сағат ішінде 37°C кезінде культураны инкубациялау. Sso7d-Taq рекомбинантты полимераза тазартылып, термиялық тұрақтылық пен элонгация уақытына ПТР әдісімен сыналды. Sso7d-Taq ферменті 95°C температурада 5 сағаттық инкубацияға және 98°C температурада 75 минуттық инкубацияға төтеп беретіні анықталды. Taq модифицирленген емес ДНҚ полимеразасымен салыстырмалы талдау Sso7d - Taq ферментінің элонгация уақытын бірнеше есе төмендететінін көрсетті: 1000 ж.н. үшін 15-13 секундқа дейін алынған нәтижелер Sso7d-Taq ДНҚ полимеразасын ғылыми зерттеулер мен диагностикалық практикада қолдану перспективасын көрсетеді.

Негізгі сөздер: ДНҚ полимераза, ПТР, *Thermus aquaticus*, Sso7d-Taq-pol, термотұрақтылық.

МОДИФИКАЦИЯ TAQ ДНК ПОЛИМЕРАЗЫ С УЛУЧШЕННОЙ СПОСОБНОСТЬЮ К ЭЛОНГАЦИИ

Сарсен А.[1,2], Акишев Ж.[1], Сагинова М.[1,2], Султанкулов Б.[1], Хасенов Б.[1]

[1]Национальный центр биотехнологии
Кургальжинское шоссе, здание 13,5, г.Нур-Султан, 010000, Казахстан
[2]Евразийский Национальный Университет им.Л.Н.Гумилева
Ул.Сатпаева, 2, г.Нур-Султан, 010000, Казахстан
khassenov@biocenter.kz

АБСТРАКТ

Термостабильные полимеразы играют важную роль в молекулярной биологии и диагностической практике. Наиболее известной и востребованной является полимераза I из термофильной бактерии *Thermus aquaticus* (Taq-pol). Эта полимераза в свое время произвела своего рода революцию в полимеразной цепной реакции. В этой работе мы попытались модифицировать эту полимеразу, присоединив дополнительный белок Sso7d из *Sulfolobus solfataricus* к Taq-pol, который обеспечивает дополнительное связывание с двухцепочечной ДНК шаблона. Ген Sso7d-Taq экспрессировали в клетках BL21(DE3). Были выбраны оптимальные условия для максимальной продукции модифицированной полимеразы Sso7d-Taq. Оптимальные условия для внутриклеточного накопления Sso7d-Taq: активация промотора T7 при достижении оптической плотности культуры OD_{600} = 0,8-1,0 путем добавления IPTG в концентрации 0,2 мм с последующей инкубацией культуры при 37°C в течение 20-24 часов. Рекомбинантная полимераза Sso7d-Taq была очищена и протестирована методом ПЦР на термическую стабильность и время элонгации. Было обнаружено, что фермент Sso7d-Taq выдерживает 5-часовую инкубацию при 95°C и 75-

минутную инкубацию при 98°С. Сравнительный анализ с немодифицированной ДНК-полимеразой Taq показал, что фермент Sso7d - Taq снижает время элонгации в несколько раз: до 15-13 секунд на 1000 п.о. Полученные результаты свидетельствуют о перспективности использования ДНК-полимеразы Sso7d-Taq в научных исследованиях и диагностической практике.

Ключевые слова: ДНК-полимераза, ПЦР, *Thermus aquaticus*, Sso7d-Taq-pol, термостабильность.