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CLONING, EXPRESSION AND PURIFICATION OF RECOMBINANT ANALOG OF *TAQ* DNA POLYMERASE

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Polymerase chain reaction (PCR) is a method that is used in solving of many molecular biology problems. At the beginning of PCR development the DNA-polymerase from *Escherichia coli* was used for amplification of DNA segments. After discovery of DNA-polymerase from *Thermus aquaticus* (*Taq*-polymerase) this enzyme due to its thermostability is widely used in PCR for DNA amplification. *Taq*-polymerase is highly thermostable DNA-polymerase. Nowadays this is the most commonly used enzyme in polymerase chain reaction and DNA sequencing. In this article we describe a procedure for obtaining the recombinant *Taq*-polymerase, including steps of cloning the gene and its expression in a heterologous environment, as well as the purification of recombinant enzyme.

Gene of *Taq*-polymerase was cloned into expression vector. The identity of cloned gene was confirmed by sequencing. Analysis of nucleotide sequence showed that recombinant *Taq*-polymerase consists of 852 amino acid residues (including 20 additional amino acids at N-terminus that contain 6xHis-tag) and has a molecular mass of 96 kDa. The recombinant protein has been purified, characterized and showed polymerase activity and thermostability.

Despite availability of a variety of commercial sources of thermostable DNA polymerases from bacteria genus *Thermus*, the most widely used enzyme is *Taq* DNA polymerase. However enzymes for molecular biology are not produced in Kazakhstan, and therefore, organization of recombinant enzymes production for use in research and diagnostic practice is an important and urgent task. The expression system and purification method used in this article allow obtaining sufficient amount of the recombinant enzyme - *Taq* DNA-polymerase.

Keywords: *Taq* DNA polymerase, polymerase chain reaction, *Thermus aquaticus*, recombinant protein.

INTRODUCTION

One of the most well-known and routine methods of molecular biology is the polymerase chain reaction (PCR). In fundamental research PCR method is used in the study of structural organization and functional significance of genes. PCR allows to do many manipulations with nucleic acids (introduction of mutations, splicing DNA fragments) and is widely used in biological and medical practice.

One of the main components of a PCR is DNA polymerase, that provides a DNA amplification [1]. Enzymes of this class catalyze the polymerization of deoxyribonucleotides along a chain of DNA nucleotides that enzyme "reads" and uses as a template [2].

At this point, one of the most actively used in laboratory practice polymerases are thermostable polymerases characterized by varying degrees of enzymological processing's speed and accuracy amplification [3]. Source of native polymerases are thermophilic microorganisms: *Thermus aquaticus* [4], *Pyrococcus furiosus*, *Pyrococcus woesei* [5], but along with natural polymerases are widely used their recombinant analogs [6, 7] and polymerases with modified structure, which ensures their low cost and improved properties .

A thermostable *Taq* DNA polymerase isolated from the thermophilic bacterium *Thermus aquaticus*, changed history of polymerase chain reaction. It has a temperature optimum of the enzymatic action of about +72°C and retains its activity at +95°C for quite a long time [8]. Considerable processivity of the enzyme (7600 nucleotides) and the high speed of

polymerization (about 60 nucleotides per second) made *Taq* DNA polymerase enzyme convenient enough for DNA sequencing.

Despite availability of a variety of commercial sources of thermostable DNA polymerases of bacteria genus *Thermus*, the most widely used enzyme is *Taq* DNA polymerase. However, enzymes for molecular biology in Kazakhstan are not made, and the organization of production polymerases sought in research papers and diagnostic practice, it is important and urgent task.

The aim of this work was the cloning, expression and purification of recombinant *Taq* DNA polymerase analog.

MATERIALS AND METHODS

Bacterial strains , DNA, plasmids and reagents

Escherichia coli DH5α strain was used for production of vectors and cloning. For the expression of heterologous protein following strains of *Escherichia coli* were used: BL21 (DE3), ArcticExpress (DE3) RP, Rosetta (DE3). DNA of *Thermus aquaticus* used as a source of DNA, was kindly provided by laboratory of biotechnology of nucleic compounds, Institute of Microbiology (Minsk, Belarus). The plasmid pET28c (+) (Novagen, UK) was used to construct the expression vector. *Escherichia coli* cells with the plasmids were cultured at +37°C in LB medium containing kanamycin at a concentration of 50 μg/ml. Enzyme Phusion High-Fidelity DNA Polymerase (Thermo Scientific, USA) was used for the amplification of the target gene - DNA polymerase with precision error rate – 4,4 x 10^{-7}. Restriction enzymes and T4 DNA ligase were purchased from Thermo Scientific (USA). For protein purification were used Ni^{2+} HisTrap HP (1 ml) and HiTrap Heparin HP (1 ml) columns (GE Healthcare, USA).

Oligonucleotides

In this paper we used oligonucleotides presented in table 1.

Table 1 - List of oligonucleotides that were used in this work

Oligonucleotide	Sequence
5*Nde*I-Taqpol	5'-GGG AAT TCC ATA TGA GGG GGA TGC TGC CCC-3'
3*Not*I-Taqpol	5'-TTT TCC TTT TGC GGC CGC TTT CAC TCC TTG GCG GAG AGC C-3'
TaqPol_706R	5'-CCC CGG GAA GGT TGT CGG ACT CGT C-3'
TaqPol_632F	5'-AGT ACG GCC TGA GGC CCG ACC AGT-3'
TaqPol_1307R	5'-CCG CCG TAG CGC CGG GCC ACC CCC-3'
TaqPol_1237F	5'-CCC ATG CTC CTC GCC TAC CTC CTG-3'
TaqPol_1237F	5'-CCC ATG CTC CTC GCC TAC CTC CTG-3'
TaqPol_1977R	5'-CAG CAC CCT GAG CTC TAT CTG GCT AT-3'
TaqPol_1897F	5'-AGG ATC CGC CGG GCC TTC ATC GCC-3'
T7fw	5'-TAA TAC GAC TCA CTA TAG GG-3`
T7rv	5'-GCT AGT TAT TGC TCA GCG G-3'

Media

We used two versions of Luria Bertani broth: normal saline for cell culturing *E. coli* DH5α/pTPh (1% tryptone, 0,5% yeast extract, 1% NaCl) and low-salt for cells ArcticExpress (DE3)RP/pTPh (1% tryptone, 0,5% yeast extract, 0,5% NaCl). For incubation of the transformed cells, SOC medium was used (2% tryptone, 0,5% yeast extract, 0,05% NaCl, 2,5 mM KCl, 20 mM $MgSO_4$, 20 mM glucose, pH 7,5).

Cloning of *Taq* DNA polymerase gene into an expression vector pET28c (+)

The PCR conditions for amplification of the target gene were: genomic DNA of *Thermus aquaticus* (100 ng) 1 μl (10 μM) of each oligonucleotide (5 *Nde*I-Taqpol primer contains a

restriction site *Nde*I, and 3 *Not*I-Taqpol primer contains the restriction site *Not*I), 4 µl (10 mM total) dNTPs, 10 µl of 5x PCR buffer (containing 7,5 mM $MgCl_2$) and 0,5 µl of Phusion High-Fidelity DNA Polymerase (2 U/µl); firstly PCR mixture was heated for 25 seconds, 30 cycles of subsequent reaction program conducted at 10 sec at +98°C, 20 sec at +57°C, 1 min at +72°C and a final elongation for 7 min at +72°C in the PCR thermocycler Mastercycler nexus gradient (Eppendorf, Germany). The amplified product was analyzed by 1% agarose gel electrophoresis with ethidium bromide. The PCR product was obtained with a corresponding size (~ 2530 bp). The amplified fragment was digested by enzymes *Not*I and *Nde*I in buffer O and then was purified by phenol -chloroform extraction and ligated into the expression vector pET28c (+), which was also treated with restriction enzymes listed above. Ligation was performed with T4 DNA ligase at +16°C for 16 hours. Highly competent DH5α cells were transformed by ligation mixture through the heat shock. Selection of colonies-transformants was performed on 1,5% LB agar with kanamycin. Total number of colonies was about 100 colonies. Next, PCR screening was performed using T7 (promoter and terminator) primers. Selected colonies-transformants were inoculated in LB broth for producing plasmid DNA. Isolation of plasmids from the positive clones was performed using a kit PureLink Quick Plasmid Miniprep Kit (Invitrogen, USA) according to the manufacturer's protocol . Check of plasmids for the presence of *Taq* DNA polymerase gene was performed using double restriction on relevant sites. Integrated plasmid insert was sequenced by the primers: T7 (promoter and terminator), TaqPol_706R, TaqPol_632F, TaqPol_1307R, TaqPol_1237F, TaqPol_1977R, TaqPol_1897F and tested for compliance with the required sequence of genomic data bank (locus TTHTAQP1A GenBank: J04639.1) using Vector NTI software Advance (TM) 11,0 (Invitrogen, USA).

Transformation and expression of a heterologous protein

Transformation of electrocompetent cells by a plasmid vector pTPh performed by electroporation using MicroPulser (BioRad, USA) under the following conditions: 100 ng of plasmid per 50 µl cell, voltage – 2,5 kV, electric capacity - 25 µF, resistance - 200 ohms. Transformed cells were incubated in 950 µl of SOC at +37°C for 1 hour with vigorous shaking. Then 50 µl of cells were plated on LB agar with kanamycin and grown at +37°C for 16 hours. Single colonies-transformants were cultured in LB broth with kanamycin in a volume of 400 ml. In the middle of logarithmic growth phase of the bacterial mass (OD600 = 0,6) inductor - isopropyl -β-D- 1 - (IPTG) was added to a final concentration of 0.5 mM and incubated for 16 hours. For analysis, samples were taken before the addition of IPTG, 2 hours after addition of IPTG and overnight culture. Collection of cells was performed at +4°C by centrifugation, 6,000 x g for 7 minutes. Further, SDS-PAGE electrophoresis was performed to determine the recombinant protein expression level. As a marker commercial marker of Thermo Scientific, SM1811 was used. The remaining volume of the cells was cultured for further purification of recombinant protein.

Lysis, chromatographic purification and SDS-PAGE electrophoresis

After culturing overnight culture was harvested by centrifugation (+4°C, 6000 x g, 7 minutes). Bacterial lysate was resuspended in buffer 20 mM NaCl, 50 mM Tris-HCl (pH 7,5), 5 ml per 1 g of the lysate. Lysing of the cells was performed using an ultrasonic disintegrator UP200S at 24 kHz pulsing regime (10 pulses, 10 seconds/pulse) on ice. Next, the lysate was incubated at +70°C for 1 hour. The insoluble fraction of the cell debris was removed by centrifugation for 1 hour at +4°C, 40000 x g.

The resulting supernatant was applied to a HisTrap Chelating HP column 1 ml, pre-equilibrated with buffer HisTrap Low Imidasol (500 mM NaCl; 50 mM Tris-HCl (pH 7,5), 20 mM Imidazole). After loading, the column was washed with 5 volumes of buffer HisTrap Low Imidasol to remove nonspecifically bound proteins.

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To find eluating concentration of imidazole a linear gradient of imidazole was used with using Buffer HisTrap Low Imidasol and HisTrap High Imidasol (500 mM NaCl; 50 mM Tris-HCl (pH 7,5), 500 mM Imidazole). Liquid chromatograph for rapid protein purification FPLC (Fast protein liquid chromatography) AKTA model Purifier10 was used in this work. Detection of protein in the fractions was performed at a wavelength of 280 nm. Fractions containing the recombinant protein Taq-pol were combined and in the combined fractions salt concentration was reduced to 50 mM NaCl and the pH was raised with 50 mM Tis-HCl (pH 8,0). Prepared combined fractions were loaded on a HiTrap Heparin HP column 1 ml, equilibrated with 3 volumes of buffer Heparin Low Salt (50 mM KCl; 50 mM Tris-HCl pH 8,0). Purification of the protein was carried out using a linear salt gradient starting with KCl buffer Heparin Low Salt Buffer and ending Heparin High Salt (1050 mM KCl; 50 mM Tris-HCl (pH 8,0). Fractions containing the recombinant protein Taq-pol, were selected for analysis by SDS-PAGE to check purity, KCl concentration in the eluates was reduced to 100 mM and all the components were added, recombinant protein was dissolved in buffer storage (100 mM KCl; 50 mM Tris-HCl (pH 8,0); 0,1 mM EDTA (pH 8,0); 0,5 mM DTT; 1% Triton X - 100 and 50% (by volume) glycerol).

Quantitative determination of the protein concentration in the fractions was determined by the Bradford assay using bovine serum albumin as a standard [9]. Electrophoretic separation of proteins was performed by the method of Laemmli [10] in 12% polyacrylamide gel under denaturing conditions. Samples were pre- denatured prior to their application to the gel at +95°C for 5 minutes. Electrophoresis was performed at 90 V for 2 hours. Staining of gel was performed using 2% Coomassie Brilliant Blue R - 250 (Sigma) in 50% ethanol, 10% acetic acid. Nonspecific binding of dye was removed with 25% ethanol and 7,5% acetic acid.

Results and discussion

One of the most efficient expression systems for producing recombinant proteins in *E.coli* is a pET- system [11]. Target genes are cloned in pET vector under the control of a strong promoter of T7 bacteriophage RNA polymerase, which provides high expression. Taq-pol gene has long length and contains within it a plurality of restriction sites, but it turned out the best combination is *Nde*I/*Not*I providing integration taq-pol gene into the vector pET - 28c (+), and the resulting protein will have an additional 20 - peptide domain hexahistidine tag. Figure 1 shows the result of taq-pol gene amplification.

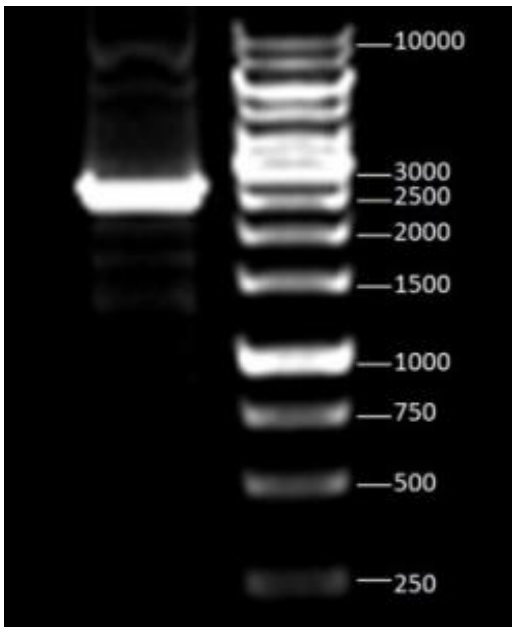

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Figure 1 - Electrophoresis in 1% agarose gel of amplified *Taq*-polymerase gene. The expected size of the PCR product is 2530 bp

PCR screening of DH5α colonies-transformants was performed for presence of vector pET-28c with the taq-pol insert by T7 primers. PCR confirmed the presence of the insert in 2 out of 9 analyzed colonies. Sequencing of plasmids from two clones confirmed the absence of mutations in the open reading frame. As a result was a genetic construction pTPh. The complete amino acid sequence of the recombinant protein Taq-pol has 852 amino acid residues with a calculated mass of 96 kDa.

During the transformation by pTPh vector following strains of *Escherichia coli* were used: BL21 (DE3), ArcticExpress (DE3) RP, Rosetta (DE3) characterized by different genotype. Comparative analysis of protein accumulation in the intracellular space showed that out of three most optimal strain is a strain BL21 (DE3)/pTPh. Induction was performed by adding isopropyl-β-D-1-thiogalactopyranoside at a concentration of 0.5 mM when the optical density of OD600=0,6. After adding the inductor, the cells were incubated at room temperature for 16 hours (figure 2).

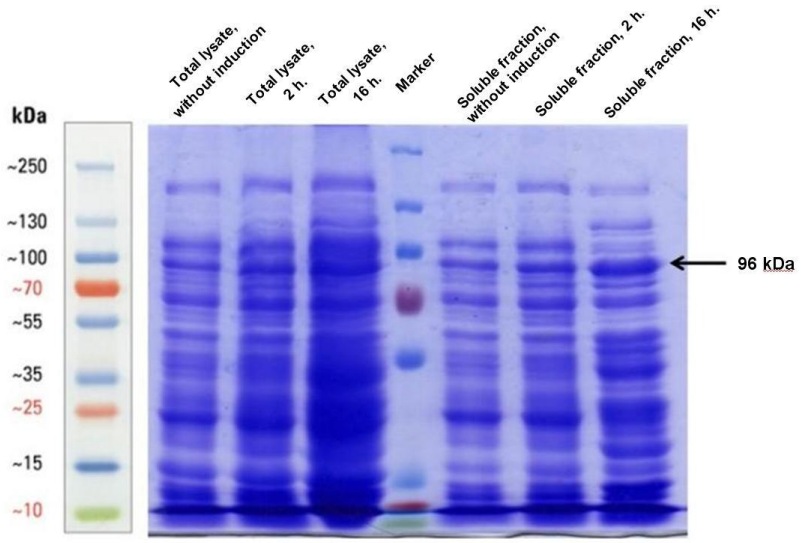

Figure 2 - Synthesis of recombinant protein TPh (pointed by arrow) in *E. coli* cells of strain BL21 (DE3) with different induction time: 2 hours and 16 hours. Calculated molecular mass of the protein is 96 kDa

As can be seen from figure 2, the results of the analysis of protein fractions show that the target protein is present in the soluble fraction and in the total lysate consisting of inclusion bodies. After 2 h, induction an increase of presence of the product corresponding molecular mass of the recombinant protein of taq-pol gene. The maximum accumulation of the target protein occurs after 16 hours of incubation.

During purification of soluble fraction of the protein lysate by metal affinity chromatography on Ni^{2+} nickel ions Fractions 24-26, corresponding to the concentration of imidazole 450 - 500 mM, contained a large number of components which absorb ultraviolet light with a wavelength of 280 nm, presumably of the target protein (figure 3a). Proteins having incorporates DNA-binding domains may be purified using biomimetric affinity ligands, such as heparin, which can be immobilized on various matrices. Structure and negative charge of heparin allow it to mimic the binding properties of DNA. After combining fractions 24-26, chromatographic purification of the protein was performed on a column of HiTrap Heparin HP (1 ml) (figure 3b).

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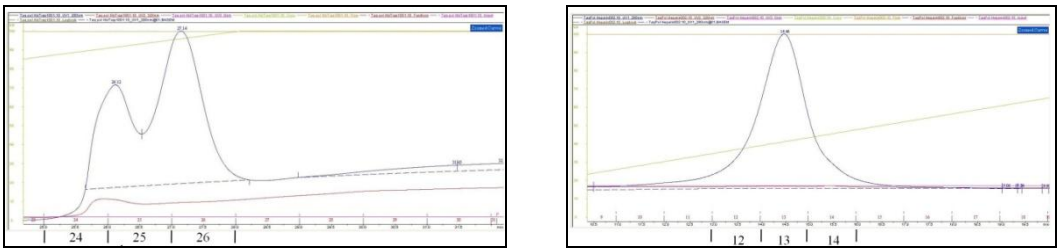

a b

Figure 3 - Chromatography for purification of recombinant protein TPh on Ni^{2+} Sepharose (a) using linear gradient of imidazole. Fractions 24-26 contain potential desired protein; (b) chromatogram for purification of recombinant protein TPh on HiTrap Heparin HP (1 ml) column

Figure 3b shows that by purification with potassium chloride as eluate, fractions 12-14, corresponding to the KCl concentration of 200 - 400 mM, contains a large amount of recombinant protein Taq-pol, which was confirmed by SDS-PAGE (figure 4).

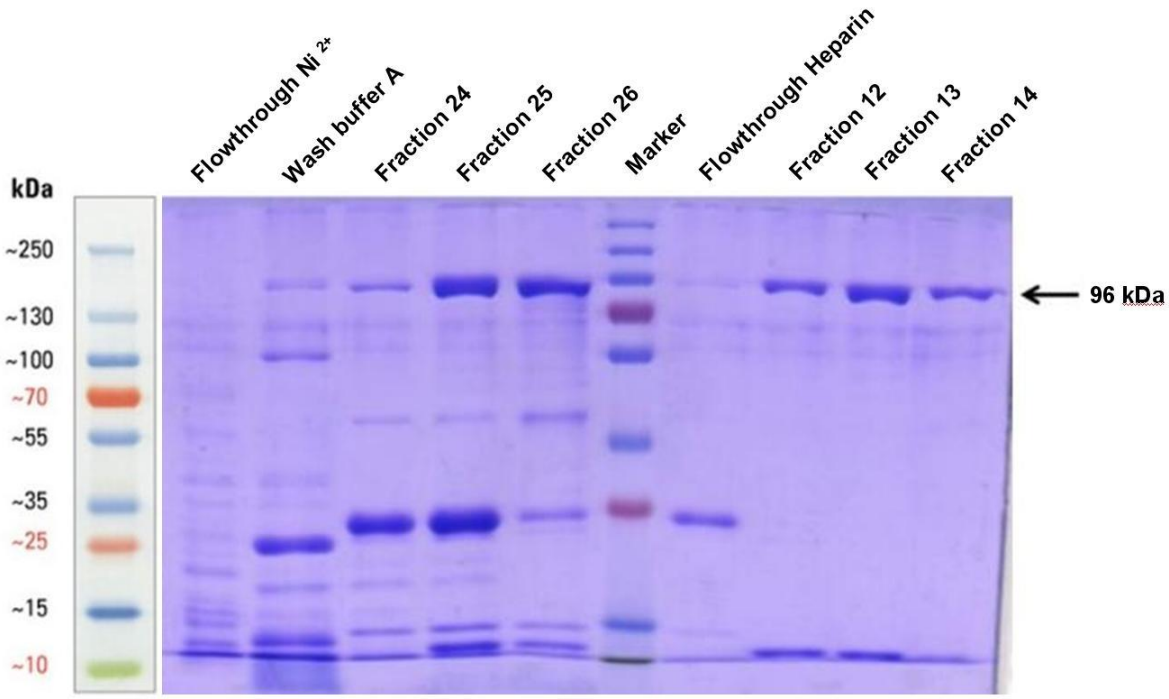

Figure 4 - Results of TPh protein purification by affinity chromatography. Samples were analyzed by 12% SDS-PAGE and stained in Coomassie blue. Flowthrough Ni^{2+} - flowthrough of the supernatant through a column containing ions Ni^{2+} Sepharose; Wash Buffer A - non specific proteins washed by buffer HisTrap Low Imidasol; fractions №24-26 - fractions № 24-26, collected during a linear gradient of imidazole with the initial buffer HisTrap Low Imidasol and final buffer HisTrap High Imidasol; Flowthrough Heparin - flowthrough of mixed fractions №24-26 through HiTrap Heparin HP (1 ml) column , fractions №12-14 - fractions №12-14, collected during a linear salt gradient with an initial buffer Heparin Low Salt and final buffer Heparin High Salt

Fractions contain almost pure protein with a molecular weight corresponding to that expected for the recombinant protein Taq-pol (96 kDa). The protein concentration in the 13^{th} sample was 300 ng/ml.

DNA polymerase activity was determined by PCR amplification of fragments of different lengths (figure 5a). Besides the expected biochemical activity resulting recombinant enzyme also showed good thermal stability over a long period of incubation at +95°C. Maximum

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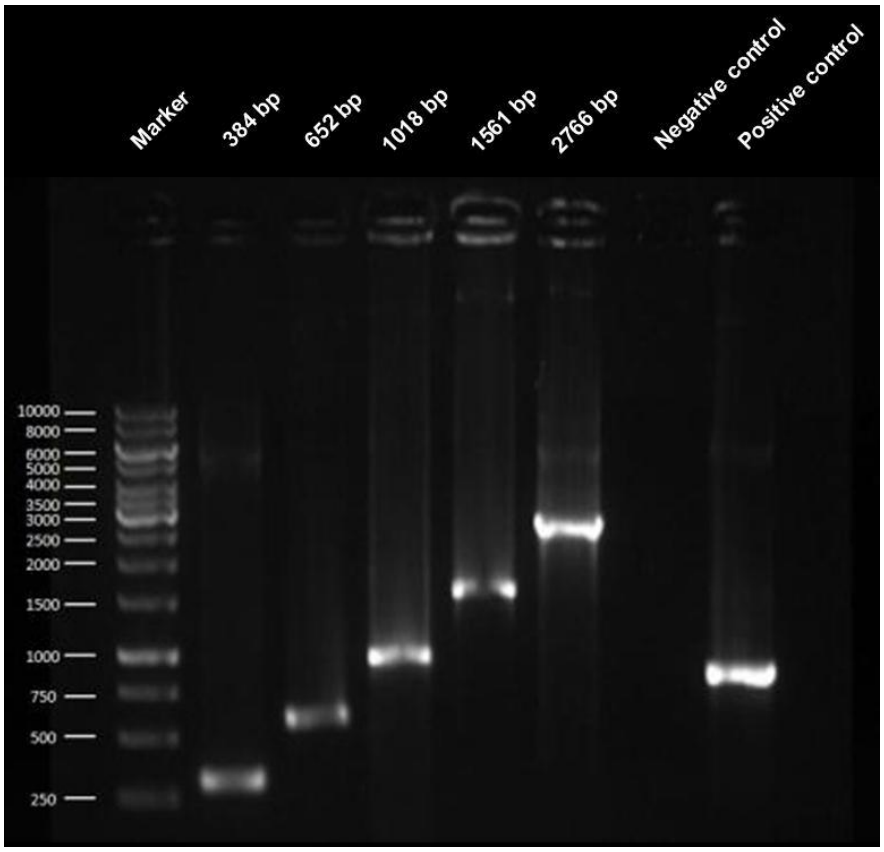
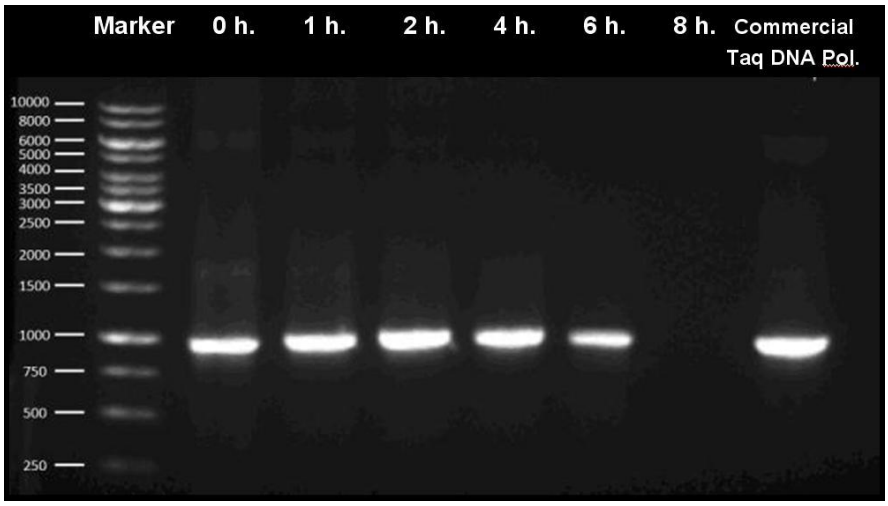
warm-up time, after which the purificated polymerase retained activity was 6 hours (figure 5b).



5a



5b

Figure 5a - The results of determination of DNA polymerase activity of TPh after purification by affinity chromatography. The length of amplified DNA fragments corresponds to the estimated size of 384 bp, 652 bp, 1018 bp, 1561 bp, 2766 bp. A commercial *Taq* DNA polymerase was used as positive control.

a

Figure 5b - The results of determination of thermal stability of TPh. The purified enzyme was warmed at +95°C during 0 hrs, 1 hr, 2 hrs, 4 hrs, 6 hrs, and 8 hrs before performing polymerase chain reaction. PCR products were analyzed on 1% agarose gel.

CONCLUSION

This article describes a procedure for obtaining the recombinant *Taq*- polymerase, including the steps of cloning a gene, its expression in a heterologous environment, and purification. *E.coli* strain BL21 (DE3) was transformed by the resulting genetically engineered construction with gene of *Taq*- DNA polymerase from the thermophilic bacterium *Thermus aquaticus* under the control of bacteriophage T7 promoter. Adding an additional 20 amino acids coding a hexahistidine tag did not affect the DNA polymerase activity and thermostability of the enzyme. The expression system and purification method allows to purify a sufficient amount of the recombinant enzyme. The purified amount of enzyme was transferred to the different laboratories of the National Center for Biotechnology for testing in various applications of PCR.

The resulting production strain was deposited in the collection of microorganisms "Scientific Research Institute of Food and Processing Industry", No. B491. The obtained results form the basis for a technological platform for obtaining recombinant enzymes for molecular biology.

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ТҮЙІН

Полимеразды тізбекті реакция (ПТР) молекулалық биология саласының көптеген мәселелерін шешу үшін қолданылатын әдіс. ПТР дамуының бастапқы қадамдарында ДНҚ сегменттерінің амплификациясы үшін *Escherichia coli*-ң ДНҚ-полимеразасы қолданылады. *Thermus aquaticus* ДНҚ-полимеразасы ашылғаннан кейін, ол өзінің термотұрақтылықтылығының арқасында ПТР-да кең қолданыла басталды. Taq ДНҚ-полимераза термотұрақты ДНҚ-полимераза. Қазіргі уақытта, бұл полимеразды тізбекті реакция, ДНҚ секвенерлеуде жиі қолданылып келе жатқан фермент. Бұл мақалада біз генді клондау, оның гетерологиялық ортада экспрессиясы және тазалау сияқты қадамдарға негізделген, рекомбинантты Taq-полимеразаны алу жолдарын сипаттадық.

Thermus aquaticus-ң ДНҚ-тәуелді Taq-полимераза гені экспрессиондық векторға клондалды. Клондалған геннің дұрыстығы секвенерлеу арқылы расталды. Нуклеотидтік тізбек Taq-полимеразаның 852 амино қышқылы қалдығынан тұратынын (қосымша 20 амин қышқылы, соның ішінде 6X His-tag туыстық домені) және молекулалық салмағы 96 кДа екенін көрсетті. Рекомбинантты ақуыз сипатталып, полимеразды белсенділік пен термотұрақтылықты көрсетті.

Thermus түріне жататын бактерияларынан алынатын коммерциялық термотұрақты ДНҚ-полимеразалардың кең ауқымына қол жетімділігіне қарамастан, Taq ДНҚ-полимераза кеңінен қолданылатын фермент. Дегенмен, молекулалық биология үшін арналған ферменттер Қазақстанда жасалмайды және де полимеразалар жасайтын мекемелер ғылыми мақалалар мен диагностикалық практикада ізделінді, бұл маңызды және аса қажетті. Бұл мақалада аталған экспрессиялық жүйе мен тазалау әдістері Taq ДНҚ полимераза рекомбинантты ақуызының жеткідікті мөлшерін тазалап алуға мүмкіндік береді.

Негізгі сөздер: Taq ДНҚ полимеразасы, полимеразды тізбекті реакция, *Thermus aquaticus*, рекомбинантты протеин.