CLONING AND PURIFICATION OF LARGE FRAGMENT OF DNA POLYMERASE I FROM *GEOBACILLUS STEAROTHERMOPHILUS* AND APPLICATION IN ISOTHERMAL DNA AMPLIFICATION

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ABSTRACT

The invention of loop-mediated isothermal amplification (LAMP) opened new research avenues in the field in diagnostics. LAMP reactions have important advantages as a diagnostic tool. These advantages include a constant reaction temperature that only requires a simple thermostat instead of a thermocycler and a greater reaction speed from 15 to 30 minutes. The method is based on a specific thermostable polymerase with helicase activity and a set of different primers. We obtained a large fragment of recombinant polymerase I from *Geobacillus stearothermophilus* expressed in *Escherichia coli*. Original strain (ATCC 12980) cells were cultivated in nutrient broth to extract genomic DNA and amplify the target gene. After its cloning and expression, the polymerase was purified in an amount of 1.5 mg from 1 L of induction culture of recombinant cells. The purified polymerase was tested and displayed polymerase and helicase activities with no exonuclease activity. These activities were demonstrated in successful LAMP reactions with the amplification of transgenic elements of genetically modified Arabidopsis thaliana using a LAMP primer set for detection of nopaline synthase terminator (T-nos) and another set for detection of the Cauliflower Mosaic Virus 35S promoter. We showed that LAMP reaction products could be detected using an agarose gel and fluorescent dyes.

Keywords: LAMP, Bst, isothermal amplification, *Geobacillus stearothermophilus*, recombinant protein, diagnostics

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INTRODUCTION

Use of thermostable enzymes, first of all DNA polymerases, has revolutionized the field of recombinant DNA technology and such enzymes are of great importance in the research industry nowadays. DNA polymerases are used for a variety of biological applications including sequencing and amplification of nucleic acids by polymerase chain reaction (PCR) requiring thermal cycling or through isothermal amplification.

A variety of thermocycling and isothermal techniques is used for amplification of nucleic acids. The thermocycling techniques use a temperature cycling to drive the repeated cycles of DNA synthesis to produce large amounts of new DNA being synthesised in proportion to the original amount of a template DNA. Another approach is to use isothermal techniques for DNA amplification that do not rely on thermocycling to drive the amplification reaction.A number of such techniques have also been developed so far. The isothermal techniques utilise DNA polymerases with strand-displacement activity and are used as a nucleic acid amplification method that can obviate the need for the repeated temperature cycles. Loop-mediated isothermal amplification (LAMP) method becomes very popular as diagnostic system for detection of various infection agents. The method was developed in 2000 by group of scientists in Japan[1]and improved in 2002 and 2008[2, 3]. The LAMP reaction mixture of various reagents is held at a constant temperature (around 65°C) and includes nucleic acid strands of the template, oligonucleotide primers, the strand displacement-type DNA polymerase and nucleic acid monomers. The LAMP assay relies on the design of a set of primers that generate stem looped (hairpin) structures during the early stage of DNA synthesis. Displacement primers help the formation of these hairpins at the ends of the DNA strands and once formed, these structures can be copied into a series of DNA fragments containing multiple units of the target sequence under isothermal conditions utilizing the displacement properties of *Bst*DNA polymerase (large fragment). Although LAMP was first described using a set of four primers, enhanced sensitivity was reported using an additional pair of loop primers.

A large number of DNA polymerases have been identified, described and shown to have varying suitability for different applications. Many DNA polymerases have also been modified in different ways such as through truncations and site-directed mutagenesis to alter their properties including alterations to abolish basic activities such as the 3'→5' exonuclease activity. DNA polymerases have been described from a number of *Thermus*species including DNA polymerase I from *Thermusaquaticus* (*Taq* DNA polymerase) which is widely used in PCR amplification due to the thermostability of the enzyme. Certain disadvantages of the PCR-based methods have been

overcome by the development of methods based on the use of strand displacement DNA polymerases. These methods have considerable advantages over other methods based on thermocycling protocols. The strand displacement methods generally produce larger fragments with higher yield and less sequence bias than PCR-based methods. The most suitable DNA polymerase that has been found and tested to date for this purpose is Bacteriophage *Phi29* DNA polymerase which has very tight binding to the DNA substrate giving very high processivity and ability to generate very long DNA products up to more than 100 kb. The essential feature of the enzyme is the ability to synthesize a new DNA strand and at the same time displace previously made DNA strands from the template strand. *Phi29* DNA polymerase originates from a mesophilic bacteriophage and the enzyme is normally used at about 30°C in an isothermal reaction, avoiding thermocycling.

The large fragment of *Geobacillus stearothermophilus* DNA polymerase is the most commonly used and most suitable DNA polymerase for strand displacement amplification without thermal cycling. The underlying ability for amplification without thermal cycling is based on strand-displacement properties of these polymerases where assumingly the DNA polymerase is able to displace annealed non-template strand and synthesize a new strand whereas conventional DNA polymerases such as *Thermus aquaticus* DNA polymerase would normally be hindered by the presence of a non-template strand annealed to the template strand. However, *Phi29* DNA polymerase is apparently not a very efficient enzyme compared to conventional DNA polymerase such as *Taq* DNA polymerase in terms of speed and thus the yield of material is produced after a certain time.

The thermostable large fragment of DNA polymerase isolated from *Geobacillus stearothermophilus*, an enzyme that is functional over the temperature range between 25 °C and 75°C, but is most active at 65°C. The *Bst*DNA polymerase (large fragment) has no 3'→5' exonuclease activity, and specifically, proof-reading 3'→5' exonuclease activity, therefore during synthesis reaction all four dNTPs are incorporated equally effectively in the chain elongation and also other nucleotide analogs, such as dITP and 7-deaza-dGTP can be included . The *Bst* DNA polymerase (large fragment) showed base pair mismatching occurs at a rate of about $1.5x10^{-5}$, that is, approximately 1.5 errors can be expected in one hundred thousand nucleotide incorporations during nucleotide polymerization catalyzed by the enzyme[4].

Isothermal amplification is possible due to specific polymerase isolated from *Geobacillus stearothermophilus*. Large fragment of this enzyme has polymerase and helicase activities with lack of 5'→3' exonuclease activity. In other words, the method is similar to helicase dependent amplification (HDA)[5] but differs from it by combining polymerase and helicase activities in one enzyme – *Bst*DNA polymerase (large fragment).

LAMP assays can be performed very quickly since there is no separate denaturation, annealing and extension steps, and as such, reactions do not require thermocyclers. Today there are many different prototypes of test systems for pathogens' detection that are based on LAMP method such as for HSV[6], toxoplasma[7], salmonella[8], mycoplasma[9, 10], listeria[11], chlamydia[12] etc. Each of them includes *Bst*DNA polymerase (large fragment) as polymerase. It explains need in producing this enzyme. We made producing strain for recombinant*Bst*DNA polymerase (large fragment), named Bstpol based on *Escherichia coli*. In this work, we describe cloning, expression and purification of this polymerase. The amplification efficiency of the LAMP primer pair sets was tested using the genomic DNA from the GMOs *Arabidopsis thaliana* lines. Bstpol successfully used for GMO testing was performed for LAMP amplified screening for the presence of GMOs using generic assays such as for 35S promoters and T-nos.

MATERIALS AND METHODS

Competent cell lines, media and DNA source

Escherichia coli cells DH5α strain was used for genetic engineering works. ArcticExpress (DE3) RP, BL-21 (DE3) and its derivative Rosetta2 (DE3) strains were used for expression of target protein. Genomic DNA of *Geobacillus stearothermophilus* strain ATCC 12980 was used for amplification. *G. stearothermophilus* was cultivated in 500 ml of nutrient broth (13g/L, peptone 5 g/L, NaCl 5 g/L, beef extract 1.5 g/L, yeast extract 1.5 g/L) at 55°C during 24 hours. Cells were collected by centrifugation at 6000×g (RCF), 4°C during 7 minutes.

Plant DNA source of template for LAMP

GM *Arabidopsis thaliana* was purchased from The Arabidopsis Biological Resource Center (ABRC) (https://www.arabidopsis.org/abrc/index.jsp).One seed was grown into a 7-day-old seedling and the leaves were collected for genomic DNA isolation. Total genomic DNA was extracted from leaf samples of these genotypes using a modification of CTAB extraction protocol (http://primerdigital.com/dna.html) with RNAse A treatment. The DNA samples were diluted in 1×TE buffer and the DNA quality was checked electrophoretically and spectrophotometrically with a Nanodrop (Thermo Fisher Scientific Inc.).

Primer design

LAMP primer sets have been synthesized based on article "Detection of genetically modified organisms (GMOs) using isothermal amplification of target DNA sequences"[13] where constitutive Cauliflower Mosaic Virus 35S promoter (CaMV 35S) and nopaline synthase terminator (T-nos) sequences were used for the design of PCR primers. The primers sequences are shown in Table 1. None of the primer form dimers, and all showed very high efficiency and for PCR and LAMP techniques. To select the best primer set, all possible primer combinations were tested for amplicon size and compatibilities. The most effective, reliable, and robust primer set is shown in table 1.

Table 1. Olygonucleotides

Primer ID	Sequence (5'-3')	Amplicon, bp	nt	Tm (°C)	CG (%)	Linguistic complexity (%)
NdeIBstpol	GGGAATTCCATATGACGGATGAAGGCGAAAAGC	1878	33	64.3	48.5	82
BamHIBstpol	CGCGGATCCGCGTTATTTGGCATCATACCATGTTGG		36	67.8	52.8	80
T-nosLampF (F1c-F2) FIP	AGATGGTTTTTATGATTAG-TTTT-ATTTATCCTAGTTTGCGC	FIP-BIP: 244 bp	41	58.3	29.3	64
T-nosLoopF (LoopFc)	CAATTATACATTTAATACGCG		21	45.8	28.6	67
T-nosDisplF (F3)	CATAGATGACACCGCG		16	51	56.3	85
T-nosLampR (B1-B2c) BIP	TAATTCAACAGAATTATATG-TTTT-AAGTTTCTTAAGATTGAATCCTG		47	56.7	23.4	71
T-nosLoopR (LoopB)	TGCAAGACCGGC		12	48.7	66.7	93
T-nosDisplR (B3c)	GATCGTTCAAACATTTGG		18	47.8	38.9	86
CaMV35P LampF (F1c-F2) FIP	GTCTTCAAAGCAAGTGG-TTTT-GGATAGTGGGATTGTGCG	FIP-BIP: 187 bp	39	65.9	46.2	77
CaMV35P LoopF (LoopFc)	TCCACTGACGTAAGGG		16	50.9	56.3	94
CaMV35P DisplF (F3)	AGGAAGGGTCTTGCG		15	52	60	87
CaMV35P LampR (B1-B2c) BIP	TTCCACGAT GCTCCTCG-TTTT-CCTCTGCCGACAGTGG		37	69.5	56.8	80
CaMV35P LoopR (LoopB)	GGGGTCCATCTTTGGG		16	54.1	62.5	76
CaMV35P DisplR (B3c)	ATAAAGGAAAGGCCATCG		18	50.3	44.4	78

Cloning large fragment of bst pol gene

A 1789 bp fragment of *Bstpol* gene was amplified from *G. Stearothermophlus* genomic DNA with 1 U Phusion Hot Star II DNA polymerase (Thermo Scientific). Assay reactions (50 ul) contained: 10µl Reaction Buffer, 4 µl2.5mM dNTPs (each), 1 µl(742 ng/µl template DNA), 10µM primers NdeIBstpol and BamHIBstpol (each). Thermocycling conditions were: preheating 98°C for 2 min, followed by 30 cycles of 98°C (15 sec), 55°C (30 sec), 72°C (2 min). Reaction was finished at 72°C for 10 min.

Obtained PCR amplicon was purified with chloroform extraction method[14]. The fragment was digested by *Nde*I and *BamH*I restriction enzymes in 2x Tango buffer at 37°C for 2 hours as well as vector pET-28c(+). Ligation was performed with T4 DNA ligase at 4°C overnight.

Competent cells DH5α were transformed with ligation mix by heat shock method. Transformed cells were inoculated on solid LB-agar 1.5% with kanamycin (50 µg/ml). PCR screening was carried out with T7-primers and Taq/Pfu DNA polymerases mix [15, 16]. Positive colonies were inoculated in liquid LB-medium in presence of kanamycin. Isolation of plasmids was performed using a Quick Plasmid Miniprep Kit (Invitrogen, USA).

Transformation of electrocompetent cells by a recombinant vector performed by electroporation method. Final recombinant vector was named pBstLF.

Expression and purification of Bstpol

Single colony was inoculated in 500 ml LB broth with 50 μg/ml of kanamycin. As soon as optical density at wavelength 600 nm was 0.6 inductor IPTG was added to final concentration 0.5 mM. 4 fractions (10 ml each) for 0, 2, 4 and 16 hours of expression were collected by centrifugation at 6000×g 4°C during 7 minutes. Expression level of target protein was examined by SDS-PAGE.

Collected fractions as well as remain culture were resuspended in buffer (20 mMNaCl, 40 mMTris-HCl, pH 7.5) and processed with ultrasonic disintegrator (50% amplitude, 0.5 cycling). Fractions were divided in 2 parts soluble and insoluble by centrifugation at 16000×g 4°C during 30 minutes.

Supernatant was heated at 60°C during 30 minutes and centrifuged at 16000×g 4°C. Purification was performed by elution with imidazole containing buffers A, B and C (20, 100 and 500 mM respectively). Preheated supernatant was loaded into equilibrated 1ml-HisTrap Chelating FF column charged with Ni^{2+}. 3 buffers with imidazole concentration: 20, 100 and 500 mM were prepared. Each buffer includes 500 mMNaCl, 40 mMTris-HCl, pH 7.5. After loading supernatant onto column elution of bound proteins was performed by subsequent addition to the column all three buffers with increasing imidazole concentration.

Eluted fractions with target protein were loaded onto 1ml-HiTrap Heparin HP column. 5 buffers with concentration of KCl: 40 mM (Buffer A), 100 mM (Buffer B), 150 mM (Buffer C), 200 mM (Buffer D) and 500 mM (Buffer E) were prepared. Each buffer includes 40 mMTris-HCl, pH 7.5.

Quantitative determination of the protein concentration in the fractions was determined by the Bradford assay using bovine serum albumin as a standard[17]. Electrophoretic separation of proteins was performed by Laemmli method[18] in 15% 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 0.2% 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.

Loop mediated isothermal amplification

Purified Bstpol protein was suspended in storage buffer: 10 mMTris-HCl (pH 7.5), 50 mMKCl, 1 mM DTT, 0.1 mM EDTA, 50% Glycerol, 0.1% Triton X-100. LAMPreactions contained 10X Isothermal Buffer: 200 mMTris-HCl (pH 8.8), 100 mM $(NH_4)_2SO_4$, 500 mMKCl, 20 mM $MgSO_4$, 1% Tween 20.

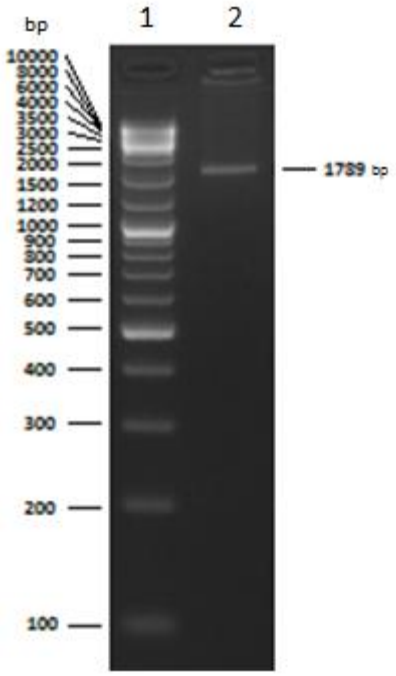
LAMP reaction was firstly performed with four inner and two outer primers. Bst 2.0 (NEB) was used as positive controls. Since reactions showed positive results with purified Bstpol as well as Bst 2.0.optimization of isothermal buffer was done. LAMP reaction was performed using set of 6 primers. Reaction was performed with constant temperature at 65°C. The composition and set-up of LAMP reaction contained: 1.6 μM FIP and BIP(each), 0.8 μM FL and BL (each), 0.4 μM F3 and B3 (each), 1X Isothermal Buffer, 0.35 mM (dNTPs each), 6 mM Mg_2SO_4, 1M Betain, 40 ng of template DNA, 3 μl Bstpol. The reaction volume was 50 μl.

RESULTS AND DISCUSSION

Obtaining of Large Fragment of DNA polymerase I from Geobacillus stearothermophilus

It is known that structure of DNA polymerase Pol I from *Geobacillus stearothermophilus* (*Bst* DNA polymerase) has a domain at its amino terminus (residues 1-310) that has 5'→3' exonuclease activity and a domain at carboxy terminus (residues 469-876) that catalyses the polymerase reaction (UniProtKB - P52026). Point mutation at position 73 (Tyr^{73}→Phe^{73} and Tyr^{73}→Ala^{73}) results in loss of enzyme 5'→3' exonuclease activity[19]. Unlike Pol I from *Escherichia coli*, the intervening domain of*Bst* DNA polymerase has lost the editing 3'→5' exonuclease activity. Structure of fragment (residues 289-876) corresponds to large fragment generated by treatment *Bst* DNA polymerase subtilisin like Klenow Fragment (Large Fragment of Pol I from *Escherichia coli*)[20]. Alignment of sequences of three large fragments from *Geobacillus stearothemophilus* (588 aa) and *Escherichia coli* (605 aa) and *Thermus aquaticus* (543 aa) demonstrates high level of homology. Bst Fragment has 55% and 56% of homology toKlenow Fragment and Taq Fragment respectively. Taq Fragment has 53% of homology to Klenow Fragment.

For Large Fragment of DNA polymerase I nucleotide sequence of *Bacillus stearothermophilus* DNA polymerase I (pol) gene was used from GenBank (L42111.1).Targeted gene locus was amplified from genomic DNA of *Geobacillusstearothermophilus*ATCC12980 (figure1).



1 – markerO'GeneRuler 1 kb DNA Ladder - 100-10,000 bp(#SM1173, Thermo Fisher Scientific); 2 – amplification of large fragment of *Bst* DNA polymerase. Expected product size is 1789 bp.

Fig. 1. Result of amplification of *bstpol* gene (large fragment)

To make LAMP reaction possible, enzyme must have no exonuclease activity at all. *Bst* DNA polymerase has 5'→3' exonuclease domain at N-end. Thereby sequence coding large fragment of *Bst* DNA polymerase with lack of 5'→3'exonuclase activity was cloned into expression vector pET-28c(+) vector under control of T7 bacteriophage RNA polymerase promoter. After amplification Large Fragment was digested by *Nde*I and *BamH*Irestriction enzymes as well as the vector. Resulting protein includes additional codons for hexa histidine-tag (6xHis-Tag) for metal-affinity chromatography step (figure 2).

MGSSHHHHHHSSGLVPRGSHMAVQTDEGEKPLAGMDFAIADSVTDEMLADKAALVVEVVGDNYHHAPIVGIALANER

GRFFLRPETALADPKFLAWLGDETKKKTMFDSKRAAVALKWKGIELRGVVFDLLLAAYLLDPAQAAGDVAAVAKMHQ

YEAVRSDEAVYGKGAKRTVPDEPTLAEHLARKAAAIWALEEPLMDELRRNEQDRLLTELEQPLAGILANMEFTGVKV

DTKRLEQMGAELTEQLQAVERRIYELAGQEFNINSPKQLGTVLFDKLQLPVLKKTKTGYSTSADVLEKLAPHHEIVE

HILHYRQLGKLQSTYIEGLLKVVHPVTGKVHTMFNQALTQTGRLSSVEPNLQNIPIRLEEGRKIRQAFVPSEPDWLI

FAADYSQIELRVLAHIAEDDNLIEAFRRGLDIHTKTAMDIFHVSEEDVTANMRRQAKAVNFGIVYGISDYGLAQNLN

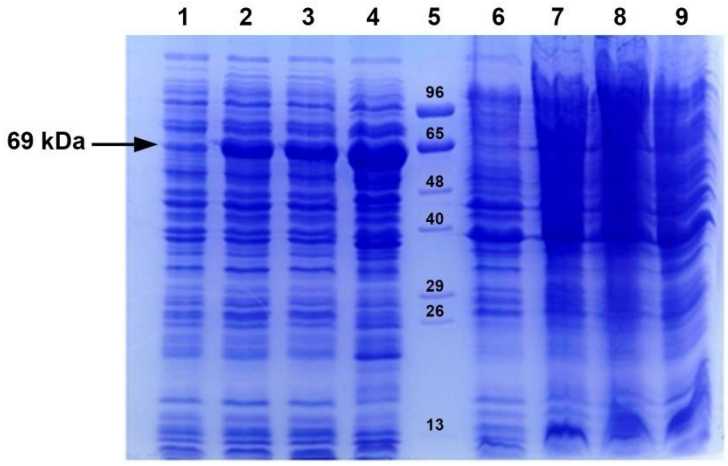
ITRKEAAEFIERYFASFPGVKQYMDNIVQEAKQKGYVTTLLHRRRYLPDITSRNFNVRSFAERTAMNTPIQGSAADI

IKKAMIDLSVRLREERLQARLLLQVHDELILEAPKEEIERLCRLVPEVMEQAVALRVPLKVDYHYGPTWYDAK

– HisTagx6; – Trombin site; – 3'-5'exonuclease domain; – polymerase domain; – DNA binding sites

Fig. 2. Sequence of recombinant large fragment of DNA polymerase I

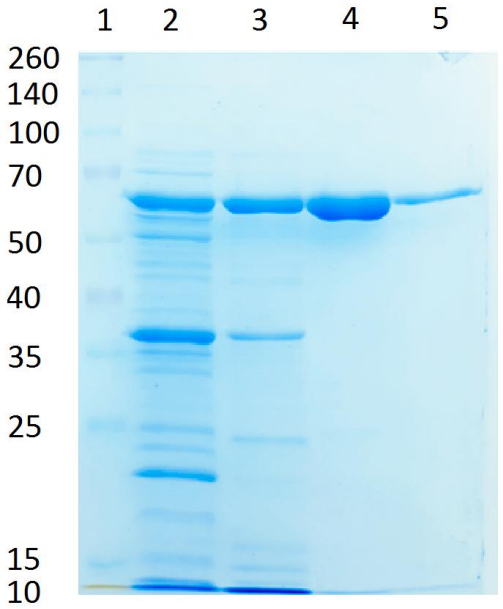
PCR screening of DH5α transformants was performed using T7 primers. As a result recombinant vector pBstLF was obtained. Recombinant protein has molecular weight of 69 kDa and length of 612 amino acid residues.Different cell lines of *E.coli* were transformed and checked for expression levels of target protein. BL-21(DE3) strain produced the highest accumulation of recombinant protein (figure 3).



1-4 – supernatant from cell lysates: 1 – 0 h; 2 – 2 h; 3 – 4 h; 4 – 16 h; 5 – protein marker; 6-9 – pellet from cell lysates: 6 – 0 h; 7 – 2h; 8 – 4h; 9 – 16h

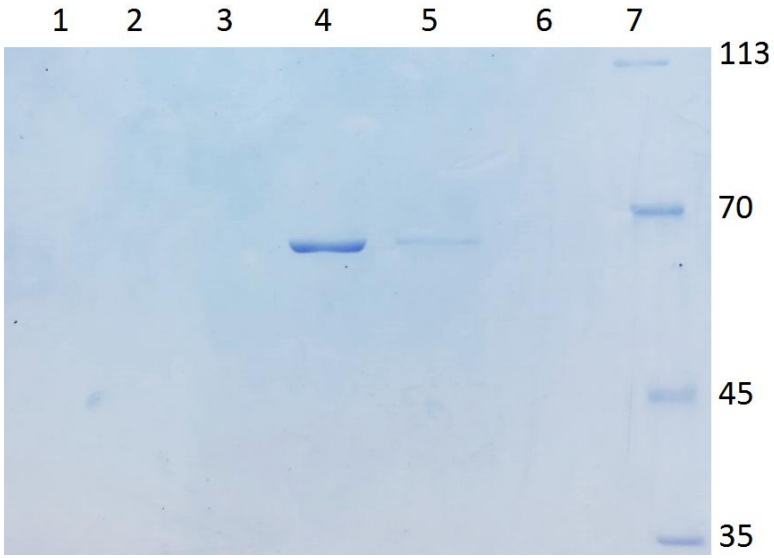
Fig.3. Expression of recombinant large fragment of *Bst* DNA polymerase in BL-21(DE3) strain of *E.coli*

According to Figure 2, it is obvious that target protein is accumulated in supernatant fraction reaching the maximum level of protein expression after overnight cultivation. This fraction was loaded onto 1 ml-HisTrap Chelating column. After purification at Ni^{2+}ions (figure 4), elutedfraction with target protein was loaded on 1 ml-HiTrap Heparin column.The bound proteins were eluted in a 40-500 mMKCl buffers (figure 5).



1– protein marker (Thermo 2634); 2 – flow through fraction; 3 – wash fraction (Buffer A); 4 – F1 fraction (Buffer B); 5 – F2 fraction (Buffer C)

Fig. 4. Metal affinity chromatography onNi^{2+} of recombinant *Bst* DNA polymerase



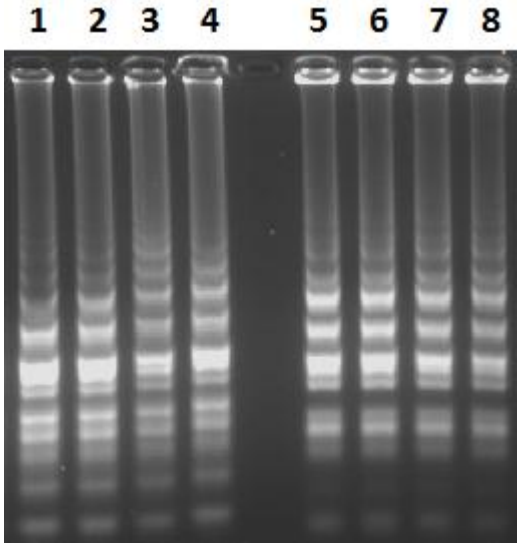
1 – flow through, 2 – wash fraction (buffer A), 3 – F1 fraction (buffer B), 4 – F2 fraction (buffer C), 5 – F3 fraction (buffer D), 6 – F4 fraction (buffer E), 7 – protein marker

Fig.5. Affinity chromatography on heparin

Figures 3 and 4 depicts all fractions from both types of purification. Figure 3 shows that the most part of protein elutes by buffer with 100 mM imidazole (lane 4 on figure 3) and by buffer with 200 mMKCl (lane 4 on figure 5). Purified protein was dissolved in storage buffer and stored at -20°C.

Loop-mediated isothermal amplification (LAMP)

The purified recombinant *Bst* DNA polymerase was tested for LAMP activity with CaMV35 primer set.



1-4 lanes – LAMP results using Bst 2.0 (NEB) with 40, 30, 20, 10 ng of GM *Arabidopsis thaliana* genomic DNA respectively; 5-8 lanes – LAMP results using recombinant Bstpol with 40, 30, 20, 10 ng of GM *Arabidopsis thaliana* genomic DNA respectively

Fig.6. Results of LAMP reactions with recombinant Bstpol

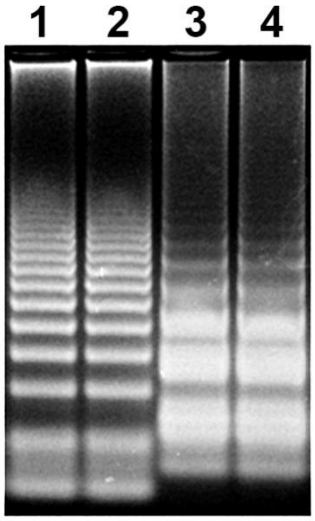
According to figure6 electrophoresis of LAMP reaction mixture with different *Bst*DNA polymerases (large fragment) shows the same results. Thus we can conclude that obtained enzyme has the same activities as Bst 2.0. It can amplify short target sequence forming loops in conditions of constant temperature. In agarose gel amplified DNA looks like ladder, which caused by different numbers of loops as well as different length of synthesized molecules.

After confirming the polymerase activity of Bstpol, the following conditions of LAMP reaction have been optimized: primers concentration, concentration of betaine and sensitivity of the LAMP reaction. Resultsshowedthat

1.2 μMconcentrationforFIPandBIP primers is enough for LAMP to work, but bands are not enough bright to make accurate conclusion. Thereby concentration of outer primers was optimized to 1.6 μM concentration. Concentration of outer primers was optimized with the same way and was 0.4 μM. Minimal concentration of template DNA was 0.2 ng/μl.Since betaine has double function, varying its' concentration could affect more than one parameters of reaction[21]. Optimal concentration of betaine was 1M.

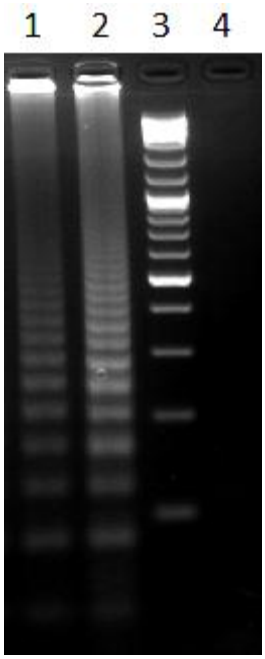
After optimizing above mentioned conditions the sensitivity of the LAMP reaction was verified by the detection of transgenic loci of genetically modified *Arabidopsis thaliana*.The most common method of GMO detection is based upon the amplification of GMO-specific DNA sequences, commonly-used motifs from transgene constructsusing PCR or LAMP methods. In this work LAMP protocol was tested for the detection of GMOs using primers that target genetic GMO sequences such as the cauliflower mosaic virus 35S promoter (CaMV 35S) and the terminator for the nopaline synthase gene (T-nos) from *Agrobacterium* spp. These sequences are commonly used in constructs used to create approved GM events. GM *Arabidopsis thaliana* contains construct both CaMV 35S and T-nos so provided convenient template for testing the assays.Using the recombinant Bstpol in the detection of GMO-specific DNA sequences yielded positive results(figure 7). Also primer sets were tested for non-specific reaction with genomic DNA of original *Arabidopsis thaliana*as matrix (figure 8).

Together with specificity and the speed at which reactions can be performed, LAMP is an excellent method for diagnostics. LAMP assay can be useful as a quick primary gel-free screen, where "positives" can then undergo a second screening such as gel electrophoresis. Here we demonstrated the sensitivity and reliability of the LAMP method for GMO detection.



1,2 – LAMP primer set for detection the nopaline synthase terminator (T-nos) (two independent reactions); 3,4 – LAMP set for detection the Cauliflower Mosaic Virus 35S promoter (CaMV 35S) (two independent reactions)

Fig. 7. Specificity of LAMP reaction on detection of transgenic elements

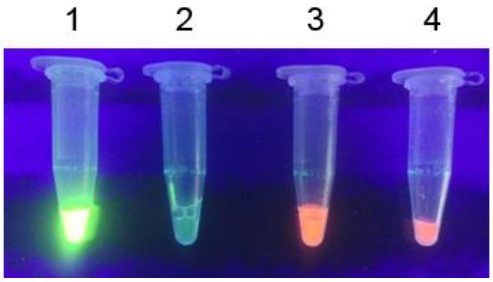


1,2 – LAMP primer set for detection the nopaline synthase terminator (T-nos) (two independent reactions); 3 – marker (Thermo SM1173); 4 – negative control (original genomic DNA of *Arabidopsis thaliana*without modifications)

Fig. 8. Specificity of LAMP reaction on detection of transgenic elements

The amplification stability of the LAMP primer pair sets was tested using the genomic DNA of GM*Arabidopsis thaliana*. Electrophoresis revealed that the unique 187bp fragment was amplified from samples containing a CaMV 35S promoter and T-nos.

LAMP method allows to detectresults in agarose gel as well as in mircotube. However, in this case instead of ethidium bromide, fluorescence dyes such as SYBRGreen or calcein are used.

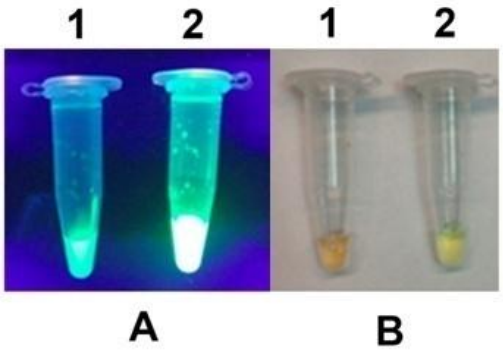


1, 2 –SYBRGreen(positive and negative results respectively); 3,4–ethidium bromide (positive and negative results respectively)

Fig. 9. Detection of LAMP results with intercalating dyes

Figure 9 shows the results of the detection of T-nos locus in genomic DNA of GM*A rabidopsis thaliana* using SYBRGreenand ethidium bromide. Results shows that ethidium bromide is not suitable for this detection since there is no significant fluorescence emission between positive and negative results. In contrast, SYBRGreen depicts great difference. These data can be explained by properties of dyes. Ethidium bromide is intercalating dye that can attach to double stranded DNA as well as to oligonucleotides which have high concentration in reaction mixture, whereas SYBRGreen reacts with double stranded DNA only.

Turbidity detection also had positive results with calcein. In the end of reaction we can observe precipitates which are pyrophosphates of magnesium[2]Using of calcein, known as calcein, allows to measure results of LAMP like other fluorescent dyes do[3].The method is based on fluorescence of calcein due to presence of ions of magnesium. There are manganese ions in reaction mixture that bind with calcein reducing its fluorescence but concentration of manganese is much lower than magnesium. Pyrophosphate ions $P_2O_7^{4-}$ are formed during amplification and bind with ions of manganese and magnesium in nearly same ratio. Since there are low concentration of manganese,calcein binds with magnesium ions which leads to fluorescence. Thus turbidity provide detection of amplification based on byproducts.



A –calcein fluorescence under UV; B – daylight;1 – negative sample; 2 – positive sample

Fig. 10. The results from the emission of calcein as a fluorescent dye in LAMP-method

As follows from the experimental results calcein presence does not affect the reaction and may be added in the preparation of LAMP-mixture and after the reaction. Fluorescence of calcein can be seen without the use of ultraviolet light (B) in household fluorescent lights lamps(figure10.).

CONCLUSION

The article describes a procedure for obtaining the recombinant large fragment of *Bst*DNA polymerase. A gene coding the polymerase was amplified from genomic DNA of *G. stearothermophilus* and cloned into expression vector pET-28c(+). *E.coli* BL-21 (DE3) strain was transformed by the resulting recombinant vector with large fragment of *Bst*DNA polymerase cloned under the control of bacteriophage T7 promoter. Recombinant enzyme has polymerase and helicase activity according to positive LAMP reactions which means that hexa histidine-tag didn't affect its activities. After purification the yield of recombinant enzyme was 1.5 mg from 1 L of induction culture of recombinant cells. The amplification efficiency of the LAMP primer pair sets was tested using the genomic DNA from the GMOs *Arabidopsis thaliana* lines. The LAMP was able to detect very small amounts of target and even in high amounts of background DNA. Bstpol successfully used for GMO testing was performed for LAMP amplified screening for the presence of GMOs using generic assays such as for CaMV 35S promoters and T-nos.

It was shown that LAMP-amplification detection results can be carried out by separating the DNA fragments in agarose gel electrophoresis, and using SYBRGreen and calceinfluorescent dyes.

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GEOBACILLUS STEAROTHERMOPHILUS-Н АЛЫНҒАН ДНҚ ПОЛИМЕРАЗА I-Ң ҮЛКЕН ФРАГМЕНТІН КЛОНДАУ МЕН ТАЗАЛАУ ЖӘНЕ ИЗОТЕРМИЯЛЫҚ ДНҚ АМПЛИФИКАЦИЯДА ҚОЛДАНЫЛУЫ.

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ТҮЙІН

Ілмекті изотермиялық амплификациясының (ИИА) ойлап табылуы диагностикада зерттеулердің кең ауқымы ашылды. ИИА реакциясы диагностикалық құрал ретінде бірнеше артықшылықтарға ие, айтарлықтай, термоциклердің орнына жай ғана термостатты қажет ететін реакция температурасының тұрақтылығы немесе 15 - 30 минут аралықта жүретін ИИА реакция жылдамдығы. Әдіс ерекше қызуға төзімді, хеликазды белсендікке ие полимераза мен әртүрлі праймерлерге негізделген. Біз *E.coli* жасушаларында экспрессияланған *Geobacillus stearothermophilusмің* I полимеразаның рекомбинантты үлкен фрагментін алдық. Бастапқы штамм (ATCC 12980) жасушалары қоректік сорпа ортасында дақылдандырылғаннан кейін, геномдық ДНҚ бөлініп, нысана ген көбейтілді. Клондау мен экспрессиядан кейін полимераза 1 литр рекомбинантты жасушалар дақылынан 1,5 мг мөлшерінде тазаланып алынды. Тазаланып алынған полимераза тексерістен өтті. Алынған фермент полимеразды және экзонуклеазалық белсендігі жоқ хеликазды белсендіктерге ие наполин терминатырының синтазасын (T-nos) және Cauliflower Mosaic Virus-ң 35S промоторын анықтауға арналған праймерлер жиынтығын қолдана отырып, генетикалық жолмен модифицирленген Arabidopsis thaliana өсімдігінің трангендік элементтерін амплификациялау арқылы сәтті ИИА әдісімен анықталды. Анықталғандай ИИА реакция өнімдері агарозды гелде және флуоресцентті бояулар арқылы анықталады.

Негізгі сөздер: LAMP, Bst, изотермиялық амплификация, *Geobacillus stearothermophilus*, рекомбинантты ақуыз, диагностика.

GEOBACILLUS STEAROTHERMOPHILUS-НАЛЫНҒАНДНҚПОЛИМЕРАЗАІ-ҢҮЛКЕНФРАГМЕНТІНКЛОНДАУМЕНТАЗАЛАУЖӘНЕИЗОТЕРМИЯЛЫҚДНҚА МПЛИФИКАЦИЯДАҚОЛДАНЫЛУЫ

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ТҮЙІН

Ілмекті изотермиялық амплификациясының (ИИА) ойланып табылуы диагностикада зерттеулердің кең ауқымы ашылды. Әдіс ерекше термотұрақты полимераза мен праймерлерге негізделген. Біз *E.coli*жасушаларында экспрессияланған *Geobacillusstearothermophilus*-ң I полимеразаның рекомбинантты үлкен фрагментін алдық. Ген *G.stearothermophilus*-ң (ATCC 12980) геномдық ДНҚ-н амплификацияланып алынды. Клондау мен экспрессиядан кейін полимераза 1 литр рекомбинантты жасушалар дақылынан 1,5 мг мөлшерінде тазаланып алынды. Тазаланып алынған полимераза тексерістен өтті. Алынған фермент полимеразды және экзонуклеазалық белсендігі жоқ хеликазды белсендіктерге ие, бұл генетикалық жолмен модификацияланған*Arabidopsisthaliana*өсімдігінің трангендік элементтерін амплификациялау арқылы сәтті ИИА әдісімен анықталды. Анықталғандай ИИА реакция өнімдері агарозды гелде және флуоресцентті бояулар арқылы анықталады.

Негізгі сөздер:LAMP, Bst, изотермиялық амплификация.