ISOLATION, CHARACTERIZATION OF NOVEL FRAGMENT OF A GENE KLENTAQ1 WHICH ENCODES THERMUS AQUATICUS DNA POLYMERASE AND ITS THERMOSTABILITY STUDIES IN *ESCHERICHIA COLI*

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ABSTRACT: Polymerase Chain Reaction (PCR) has become a powerful method for the identification and amplification of genes for direct sequencing and clinical diagnosis. In the present study an attempt was made to isolate an enzyme with widespread usage in PCR which can work in extremely high temperatures without comprising its biological activity. However, researchers have been trying to produce such enzyme from various versions of Taq DNA polymerase gene via genetic engineering. The present work reports the extraction of two novel genes encoding the enzymes which play important roles in regulating DNA metabolism with respect to the temperature. The "klentaq1" part (polymerase domain) of the enzyme was cloned with polymerase activity using specific primers. Then, a suitable cloning vector ("pET21 a") was used for its transfer to E. coli BL21. The enzyme was then studied in the presence of Isopropyl β-D-1-thiogalactopyranoside and purified. Thermo-stability of the enzyme was compared with that of commercially available Taq polymerase. Two genes have been newly registered in the gene bank (Genbank Reference No.: EU682501, Version: EU682501.1 and AM999769.1.) and defined as Thermus aquaticus 16S ribosomal RNA gene with 1430 bp. The product of the gene, Taq DNA polymerase was also isolated. The comparative results of thermo-stability between the isolated enzyme and the commercial one at 95 ºC for 30 min period revealed that the isolated klentaq1 DNA polymerase showed 75% more biological activity than that of the commercial enzyme. In addition, cloned fragment of the enzyme showed higher thermo-stability than the wild type enzyme.

Key words: *Klentaq*1, cloning, PCR, *E. coli*, DNA plymerase, vector, thermo-stability.

1. INTRODUCTION

DNA amplification using Polymerase Chain Reaction (PCR) is an essential requirement for genetic analysis. The discovery of the thermostable DNA polymerase from *Thermus aquaticus* (*Taq*) has brought the revolution in genetic engineering. *Taq* DNA polymerase isolated from *Thermus aquaticus* has high optimum temperature activity and thus it has more advantages than the thermo-labile DNA polymerase from *Escherichia coli* (*E. coli*) [1]. At higher temperature primer binding is more specific which results in higher yield of the desired products as well as less amplification of nonspecific products. Moreover, at the temperature required to denature the duplex DNA products, the DNA polymerase-I from *E. coli* is mostly inactivated. Due to the stability of *Taq* DNA polymerase at high temperature (93-95 ºC), it can be added at beginning of the PCR reaction rather than adding at the start of every cycle of amplification [2].

It is reported that the *Taq* DNA polymerase lacks 3`-5` exonuclease activity and therefore, it is unable to cleave the mis-incorporated nucleotides [3]. This indicates that, *Taq* DNA polymerase is substantially susceptible to error-prone DNA amplification. It is shown that, mutations in proteins with N-terminal deletion lead to reduced tendency toward errors, simillarly several recent studies reported about the latest discoveries on the thermo-stable DNA polymerase with an integral editing exonuclease activity [4]. Nevertheless, even such enzymes cannot amplify the sequence in excess of 5.0 to 7.0 kb, whereas *Taq* DNA polymerase can readily amplify the larger DNA fragments even upto 35 kb. This advantage of *Taq* DNA polymerase can be achieved by combining the *klentaq1* DNA polymerase (N-terminal deleted *Taq* DNA polymerase) with a thermostable DNA polymerase having 3`-5` exonuclease activity [5].

It is well known that, the DNA sequencing essentially required *Taq* DNA polymerase and enzymes with N-terminal deletion [6]. *Taq* DNA polymerase gene with 2499 base pairs is estimated to encode a protein of 832 amino acids [7]. However, due to the extremely less affinity of the enzyme towards di-deoxy nucleotide, very little scientific data is available on *Taq* DNA polymerase. Therefore, research on a suitable mutant of the enzyme with high affinity for di-deoxy nucleotide, has become considerably interesting and important [8]. With this background, in the present study an attempt was made to isolate, purify and characterize *klentaq1* DNA polymerase and to study the expression of the gene for *klentaq1* DNA polymerase. In addition, comparison of thermo-stability of *klentaq1* DNA polymerase with commercial *taq* DNA polymerase was evaluated.

2. METHODOLOGY

2.1 Bacterial Strains and Culture Conditions

Strain of *Thermus aquaticus* was obtained from Applied Biosystems. *Escherichia coli* strain (*BL21*) maintained at our laboratory, was used as the host for plasmid preparation. *Thermus aquaticus* cells were grown in tryptase agar medium at 75ºC and *Escherichia coli* cells were grown in Luria-Bertani medium at 37ºC. The plate was solidified with 1.5% (w/v) agar; ampicillin (100 µg/ml) was added in the culture medium when necessary.

2.2. Extraction of total DNA of *Thermus aquaticus* (*Taq*)

Three kinds of buffer solutions were used for the extraction of *Taq* DNA. Briefly, *Taq* bacterial were cultured on the agarose plate and were incubated at 37ºc for 24 hours followed by collection of cells from culture medium and washed with distilled water. 1 mL of buffer (50 mM EDTA, 50 mM Tris-base pH= 8) was added to the sediment and cells were kept in -20 ºC for 1 hour. 100µl of solution 2 (10 mg/ml lysozyme in 0.25 M Tris-Base at pH= 8) was added to the frozen bacteria and kept in room temperature for 45 minutes. Then 200 µl of solution 3 (10 mg/ml proteinase K, 5% SDS, 50 mM Tris-Base at pH=7.5, 0.4 EDTA) was added and sample was centrifuged in 12000 rpm for 10 minutes. The lower layer of phenol was extracted then an equal volume of phenol and chloroform was added followed by centrifugation at 12000 rpm for 10 minutes. 1/10 volume of sodium acetate 3 M was added and mixed slowly followed by addition of 100% Ethanol with gentle shaking. Samples were kept in -20 ºC for 1 hour and then centrifuged and supernatant was discarded. Sediment was washed with ethanol 70% twice and kept at room temperature to evaporate the alcohol. The sediment was then dissolved in 50 µl TE buffer or double distilled water, the gel electrophoresis was run to confirm the integrity of extracted DNA [9].

2.3. Molecular Cloning of the Gene for *klentaq1* DNA polymerase

The reported methods with little modification was adapted for isolation of genomic DNA of *Thermus aquaticus* and plasmid DNA, followed by preparation of *klentaq1* fragment gene with 1650 bp by PCR amplification using the *Thermus aquaticus* genomic DNA. The sequence of forward and reverse primers used for the amplification were given in the Table 1 which created the underlined unique sites *EcoRI* and *SalI* restriction enzyme respectively, at each end of the amplification DNA fragment. Following the digestion of amplified product by *EcoRI* and *SalI*; the 1.65 kb DNA fragment was isolated from the low-melting point agarose gel. The digested fragment was then ligated into an expression vector *pET 21a*. A fusion was created using lac promoter and ligate was transformed in to *E. coli* (*BL21*) [10].

2.4. Culture and Expression Conditions

Briefly, 400 µl of sample of an overnight culture of *E. coli* (*BL21*) harboring the recombinant plasmid *pET21 a-klentaq1* was grown in Luria-Bertani broth containing ampicillin and was transferred to 20 ml of terrific broth and the culture was incubated at 37ºC. To obtain the A600 of the culture of 0.6, the culture was induced by the addition of 0.2 mM Isopropyl -D- thiogalactoside (IPTG) and then incubated at 37ºC for 4 hours.

2.4.1. Extraction, Purification and Determination the Expressed *klentaq1* DNA polymerase I in *E. coli*

The cell was harvested by centrifugation. The cell pellet was resuspended in buffer A (50 mM Tris-HCl pH 7.9, 1 mM EDTA), then centrifuged in 6000 rpm for 10 min. The pellet was re-suspended in buffer B (4 mg/ml lysozyme in 50 mM Tris-HCl pH 7.9, 1 mM EDTA) and kept at room temperature for 15 min. Then 1 ml buffer A was added. 1 ml buffer C (10 mM Tris-HCl pH 7.9, 50 mM KCl, 1 mM EDTA, 100mM PMSF, 0.5% Tween 20, 0.5% Triton X100); then the solution was incubated in a water bath at 75ºC for 30-60 min to denature *E. coli* proteins. Denatured proteins were removed by centrifugation 8000 rpm for 10 min, then the supernatant containing *klentaq1* was analyzed and assayed [11]. Primers and PCR conditions for searching optimal condition of PCR Oligonucleotide primer that annealed to 16s rDNA was designed to give 1500 bp amplified DNA fragments. PCR mixture (50 µl) containing 20 p moles of primers, each dNTPs at 200 mM, 1 unit of *klentaq1* DNA polymerase, and bacterial DNA was used. After a single 5 min denaturation step at 95ºC, PCR (35 cycles) was done by 1 min of denaturation at 95ºC, 1 min of annealing at 58ºC, and 2 min of extension at 72ºC. A final 10 min extension at 72ºC was performed before termination of the reactions [12].

2.4.2. Sodium Dodecyl Sulphate-Polyacrlyamide Gel Electrophoresis (SDS-PAGE)

SDS/PAGE was performed with 12.5% (w/v) polyacrylamide in a vertical slab gel apparatus as described by Laemmli. Protein samples (Tris buffer extraction) were then loaded into the wells of polymerized gel. Electrophoresis was performed at a constant voltage of 200 volts when samples were in the stacking gel. At completion of electrophoresis, the glass sandwich was disassembled. The stacking gel and the resolving gel were discarded. Molecular weights of the proteins were determined by comparing relative mobility of protein bands to the standard protein markers. Protein bands intensity analysis was carried out using the Total Lab [13].

Table 1: The sequences used for primer

Direction	Nucleotide Sequences
Forward	5'-TTTGAATTCATGCTCCTC CACGAGTTCGGCCTTC-3'
Reverse	5'-AAAGTCGACGGTGGTATC ACTCCTTGGCGGAGAG-3'

3. RESULTS AND DISCUSSION

DNA of *E-coli* was extracted using phenol/chloroform assay. Intensity of extracted DNA was 0.71% µg/ml. For quantification of extracted DNA the samples were analyzed at two wave lengths i.e. 260 and 280 nm and the samples having proportion between 1.8 and 2 were selected and used for PCR (Figure 1).

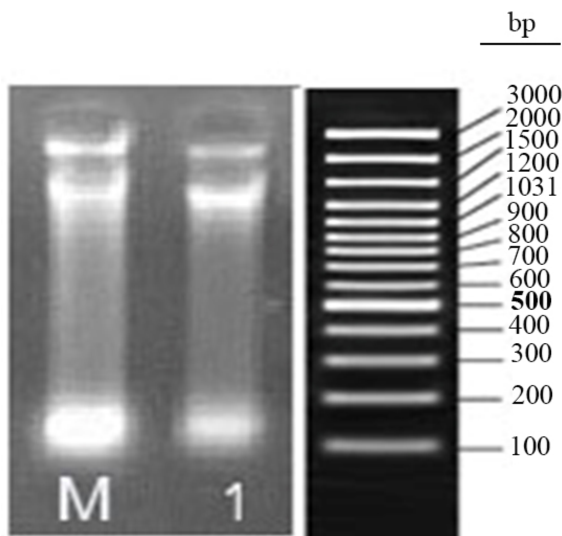

Figure 1: Gel Electrophoresis of Extracted DNA on 0.7% Agarose Gel.

The purification of the enzyme was monitored by SDS/PAGE using 12.5% polyacrylamide gel. Almost all the proteins originating from *E. coli* were removed by heat treatment. The purified sample of *klentaq1* DNA polymerase seemed to be homogeneous by SDS/PAGE. A single protein band corresponding to a molecular mass of 64 kDa was obtained by SDS/PAGE showing good agreement with 64 kDa calculated from the deduced amino acid sequence (Figure 2).

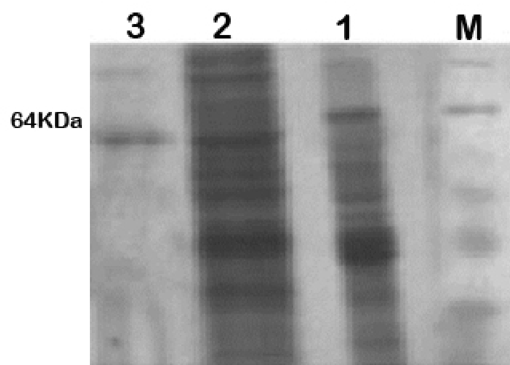

Figure 2: SDS/PAGE analysis of klentaq1 DNA polymerase (1=Uninduced cell, 2= Sonicated extract, 3= Heat treatment and 4= Low molecular mass number).

Effect of temperature on the activity and stability of *klentaq1* DNA polymerase activity was determined at 95ºC for 2 hours. The enzyme was fairly stable up to 72 ºC, at temperature above 95 ºC, the thermo-stability of the enzyme decreased drastically. The half-life at 95ºC was approximately 45 min while the half-life of wild type *Taq* DNA polymerase was approximately 25 min.

DNA polymerase obtained from *T. aquaticus* has been using widely in genetic engineering for DNA amplification and DNA sequencing protocols. Furthermore the stability of *Taq* DNA polymerase in PCR at higher temperature has made it possible to proliferate DNA between multiple cycles and thus can be used at higher temperature (>72 ºC) for attaching the primers with greater accuracy [17,19]. The current work reports the extraction of two novel genes encoding the enzymes which play important roles in regulating DNA metabolism with respect to the temperature. These genes have been newly registered in the gene bank (Genbank Reference No.: EU682501, Version: EU682501.1 and AM999769.1.) and defined as *Thermus aquaticus* 16S ribosomal RNA gene with 1430 bp. Their functional biochemistry has become an integral part of the PCR method which was developed to replicate a DNA sequence. These two genes distinguish *Thermus aquaticus* from other thermophiles, thus were isolated and cloned in a different host and their expressed enzymes were compared with a wild type. The production of thermo-stable enzymes in the *E. coli* system has advantages that not only it can be produced in large quantity but also the ease of purification by using different physical properties of the thermo-stable enzyme compared with those of the host proteins. In this study these benefits were exploited in the purification of *klentaq1* DNA polymerase in *E. coli. Taq* DNA polymerase enzyme which has missing amino acids at position 280 from its N-terminal end region. *Klentaq 1* also has a lesser tendency to cause transcription error due to its proofreading function. It is more thermo-stable in comparison to *Taq* DNA polymerase enzyme at 99 ºC and can also be used for DNA sequencing [14,15].

PCR is an effective technique for recognition and proliferation of genes, sequencing and clinical diagnosis. There are a lot of special usages of PCR in clinical diagnosis such as recognition of new mutations, monogenic disease and analyzing of biologic observations, recognition and diagnosis of oncogenes and human infectious diseases [16, 17, 18].

The goal of this work was to produce an enzyme with a wide range of application in PCR which can retain its biological activity especially at higher temperature. Its thermo-stability also implies that it can be used in small amounts thus being cost effective.

Given the advantages and its special characteristics, in the current work, we induced the proliferation of *Klentaq1* fragment of *Taq* polymerase enzyme, cloned and finally transferred it to the *E-coli* bacteria primers [17]. For/ Rev *Klentaq1* and For/ Rev *Taq* were employed which during PCR was cut with two restriction enzyme *EcoR1* and *Sal1*, cloned in plasmid *PET21*a and transferred to *E. coli* bacteria. *Klentaq1* expression in the presence of IPTG was then prepared. This enzyme was isolated by heat extraction technique. Also the results of SDS- PAGEs showed just some trace of impurities (a thin band more over than protein band). Furthermore the concentration of expressed protein by Bradford test showed the total amount of protein was evaluated 0.55 µg/mL, but the thermo-stability activity of at 93 ºC was compared with a commercial enzyme. Our results show that after 30 minutes the enzyme retain 75% of its biological stability while its similar commercial counterpart had less than 25% activity. Although these results are positive and reproducible more robust stability studies need to be undertaken to rule out other unknown practical issues which may affect the efficiency of this system. Nevertheless it is clear in our study, *klentaq1* DNA polymerase has high

thermo stability compared to the *Taq* DNA polymerase. It is thus likely that *klentaq1* DNA polymerase could be useful for use on a PCR sample with high percent of Guanosine-Cytosine and high temperature.

The extreme importance of thermo-stable DNA polymerases for biotechnology has propelled the increasing interest in seeking and characterization of new DNA polymerases of Thermophiles. The current study introduces a new and highly thermo-stable system (*klentaq1*) that has potential use in PCR methods. This warrants further research into its production, efficiency, stability and other comparative studies with available systems on the market to full determine its advantages. Furthermore our data support the use of thermophile bacteria to produce thermo-stable enzyme system [19]. This potentially can be a more rapid and economically viable approach to be employed in a commercial setup or for up scaling purposes.

4. CONCLUSION

In the present work, two genes isolated and characterized as *Thermus aquaticus* 16S ribosomal RNA gene with 1430 bp. *Taq* DNA polymerase, a product of the genes was also isolated. The isolated enzyme, *Taq* DNA polymerase, was found to be more thermo-stable than the commercial one. The thermo-stability of the isolated enzyme compared to the commercial one at 95ºc for 30 min showed that *Taq* DNA polymerase proved to be 75% better than the commercial one with respect to the enzymatic activity. The cloned fragment of enzyme showed higher thermo stability than the wild type enzyme. Thus it can be concluded that, *T. aquaticus* 16S ribosomal RNA gene with 1430 bp could be a better source to produce remarkably more thermo-stable *Taq* DNA polymerase using in *E-coli*.

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