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Family B DNA polymerase from a hyperthermophilic archaeon *Pyrobaculum calidifontis*: Cloning, characterization and PCR application

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The 2352 bp gene coding for 783 amino acid family B DNA polymerase from *Pyrobaculum calidifontis* was cloned and expressed in *Escherichia coli*. Expression of the gene resulted in the production of *Pca*-Pol in soluble fraction. After heat denaturation of the host proteins, the *Pca*-Pol was further purified by ion exchange and hydrophobic interaction chromatographies. Activity gel analysis showed the presence of a catalytically active polypeptide of about 90 kDa. The mass of the protein, determined by Matrix-Assisted Laser Desorption/Ionization-Time of Flight Mass Spectrometry was found to be 89,156 Da. The isoelectric point of the enzyme was found to be 6.13. The optimal pH and magnesium ion concentration for the enzyme activity were 8.5 and 4 mM, respectively. Unlike other commercially available DNA polymerases the enzyme activity of *Pca*-Pol was inhibited by monovalent cations such as ammonium and potassium. The half-life of the polymerase at 95°C and 100°C was 4.5 h and 0.5 h, respectively. The enzyme possessed 3′→5′ exonuclease activity and was able to amplify, under suitable conditions, up to 7.5 kb DNA fragments by polymerase chain reaction which makes it a potential candidate for amplification of long DNA fragments.

[**Key words:** Archaea; DNA polymerase; Hyperthermophile; *Pyrobaculum calidifontis*; Exonuclease activity; Polymerase chain reaction]

The domain archaea constitutes the third major branch of living organisms (1). Archaea are divided into four phyla (2,3) namely Crenarchaeota, Euryarchaeota, Korarchaeota and Nanoarchaeota. Many of the crenarchaeotes appear to be hyperthermophiles (organisms growing optimally at 80°C or above). Because of their heat tolerance, proteins from hyperthermophiles are of significant importance (4).

DNA directed DNA polymerase (E.C. number 2.7.7.7) plays an important role in cellular DNA replication and repair. Based on amino acid sequence homology, DNA polymerases have been classified into families A, B, C, D, E, X and Y (5–8). Archaea are known to possess family B and D DNA polymerases (9). The members of crenarchaeota possess family B DNA polymerases (10) whereas euryarchaeotes contain both family B and D DNA polymerases (11,12).

Thermostable DNA polymerases are required in polymerase chain reaction (PCR). PCR is an important method used for DNA amplification, sequencing, diagnosis and site directed mutagenesis (13). DNA polymerase from *Thermus aquaticus* was the first thermostable DNA polymerase to be characterized (14). However, it has no 3′→5′ exonuclease activity which is responsible for the proofreading ability of the enzyme. Archaeal DNA polymerases are known to possess 3′→5′ exonuclease activity and hence have better fidelity as compared to that of *Taq* DNA polymerase.

Pyrobaculum calidifontis is a hyperthermophilic archaeon isolated from hot water spring in the Philippines. *P. calidifontis* is a rod shaped, heterotrophic crenarchaeote that grows optimally at 90°C to 95°C and pH 7 in atmospheric air (15).

In this study we describe gene cloning and expression of a thermostable family B DNA polymerase from *P. calidifontis*. The recombinant enzyme was purified and various biochemical properties were studied. Application of the polymerase in PCR was also demonstrated.

MATERIALS AND METHODS

Strains, enzymes, vectors, chemicals *P. calidifontis*, used as the donor organism for DNA polymerase gene, was isolated by our group at Kyoto University, Japan (15). *Escherichia coli* DH5α, *E. coli* BL21 CodonPlus(DE3)-RIL and expression vector pET-21a(+) were from Novagen (Madison, USA), TA cloning vector pTZ57R/T, all enzymes, deoxyribonucleoside triphosphates (dNTPs), dideoxyribonucleoside triphosphates (ddNTPs), biotin chromogenic detection kit, gel extraction kit and GeneJet plasmid isolation kit were purchased from Fermentas (Ontario, Canada). Bradford reagent was purchased from Bio-Rad (Hercules, CA, USA). Sephadex G-25 spin columns were from GE Healthcare (Piscataway, NJ, USA). TTP [methyl-^{3}H] was from MP Biomedicals (Solon, OH, USA). IPG strips from Serva (Reno, NV, USA) were used for determination of *pI*. Agarose, aphidicolin and activated calf thymus DNA were from Sigma (St. Louis, MO, USA). Oligos (unlabelled and biotinylated) were synthesized commercially by GeneLink (Hawthorne, NY, USA). Medium components for growth of *P. calidifontis* were from Nacalai Tesque Inc. (Kyoto, Japan).

Growth of *P. calidifontis* and genomic DNA isolation Growth medium for *P. calidifontis* contained 1% tryptone, 0.1% yeast extract and 0.3% sodium thiosulfate. Glycerol stock of *P. calidifontis* was inoculated (1%) in 25 ml of medium and grown for 24 hours at 90°C with shaking at 100 rpm. This pre-culture was used to inoculate 200 ml growth medium. After growth of two days under same conditions, cells were harvested by centrifugation at 6500 rpm for 15 min at 4°C. Genomic DNA isolation and other DNA manipulations were done using standard procedures (16).

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PCR amplification and cloning of the polymerase gene Genomic DNA of *P. calidifontis* was used as a template for PCR amplification of DNA polymerase gene (*Pca*-pol). Genome sequence revealed the presence of two family B DNA polymerases, one belonging to each subfamily B1 and B3. Based on the sequence of the polymerase gene of family B3 (accession no. CP000561), two primers Pol2N (5′-CATATGAGGTTTTGGCCTCTAGACGCCACGTACTCTG-3′) and Pol2C (5′-GCAGAAACTAGCCTAGGAAGTCCAAGAGTG-3′) were synthesized. The forward primer Pol2N contained an *Nde*I recognition site (shown underlined). PCR was performed by using *Taq* DNA polymerase. The PCR conditions were: initial denaturation step at 95°C for 2 min; followed by 30 cycles of: denaturation at 95°C for 30 s, annealing at 54°C for 30 s, extension at 72°C for 90 s; and a final extension step at 72°C for 10 min. Electrophoresis of the PCR product was carried out and the amplified product was eluted from gel. This gel purified DNA polymerase gene was ligated to TA cloning vector pTZ57R/T (Fermentas) and transformed *E. coli* DH5α. This recombinant plasmid was named pol-pTZ57. This plasmid was then digested with *Nde*I and *Bam*HI to liberate the polymerase gene which was gel purified and ligated to pET-21a(+) (Novagen) digested with the same restriction enzymes. The resulting recombinant expression vector was named pol-pET21a.

DNA sequencing and sequence analysis After cloning in expression vector, the presence of *Pca*-pol was confirmed by DNA sequencing using CEQ800 Beckman Coulter sequencing system. Multiple sequence alignment was performed by using ClustalW programme (17).

Expression of *Pca*-pol gene and purification of recombinant protein *E. coli* BL21 CodonPlus(DE3)-RIL cells were transformed with expression vector pol-pET21a and grown in LB medium. Gene expression was induced by the addition of 0.2 mM isopropyl-β-D-thiogalactoside (IPTG) at an OD_{600} of 0.4, followed by further incubation for 4 h at 37°C with shaking at 100 rpm. Cells were harvested by centrifugation at 6500 rpm for 15 min at 4°C. The cell pallet was suspended in 25 mM Tris–Cl pH 8.5 and lysed by sonication. After centrifugation at 15,000 rpm for 15 min at 4°C, the supernatant obtained was heated at 80°C for 30 min and centrifuged. Nucleic acids were precipitated by addition of polyethylenimine (0.3% final concentration) followed by centrifugation. The resulting supernatant was subjected to anion exchange chromatography by using ÄKTApurifier FPLC system (GE Healthcare). The anion exchange column (ResourceQ-6 ml) was equilibrated with 25 mM Tris–Cl pH 8.5 and the crude enzyme preparation was loaded onto the column. Proteins were eluted by a linear gradient of 0 to 1 M NaCl in equilibration buffer. Major fractions containing the *Pca*-Pol were collected and dialyzed against 25 mM Tris–Cl pH 8.5. After dialysis, $(NH_4)_2SO_4$ was added to protein solution (1 M final concentration) and loaded onto hydrophobic interaction column (ResourcePHE-1 ml) equilibrated with 25 mM Tris–Cl pH 8.5 containing 1 M $(NH_4)_2SO_4$. *Pca*-Pol was eluted by a linear gradient of 1 to 0 M $(NH_4)_2SO_4$ prepared in 25 mM Tris–Cl pH 8.5. The fractions containing *Pca*-Pol were dialyzed against the storage buffer (25 mM Tris–Cl pH 8.5, 0.1% Tween 20, 0.2 mg/ml BSA and 50% glycerol) and stored at −20°C.

DNA polymerase activity gel analysis DNA polymerase activity gel analysis was performed as described in literature by Kahler and Antranikian (18) with slight modifications. All buffers contained 25 mM Tris–Cl pH 8.5, $MgCl_2$ was used at a concentration of 4 mM, the labeled nucleotide was biotin-11-UTP and 2U purified *Pca*-Pol was used for activity gel analysis. *Taq* DNA polymerase and Klenow fragment (1U each) were used as positive and negative controls, respectively. After transfer of DNA to Hybond N+ membrane (GE Healthcare), detection of incorporated biotin-11-UTP was done by using biotin chromogenic detection kit (Fermentas) according to the instructions of the manufacturer.

Determination of molecular mass and isoelectric point Molecular mass analysis of the recombinant protein by Matrix Assisted Laser Desorption Ionization-Time of Flight Mass Spectrometry (MALDI-TOF MS) was performed. The purified enzyme was desalted by Sephadex G-25 column (GE Healthcare). 1 µl of salt free protein solution (5 mg/ml) was mixed with 10 µl of 3,5-dimethoxy-4-hydroxycinnamic acid (10 mg/ml) prepared in 1/3 of acetonitrile, 2/3 of 0.1% trifluoroacetic acid (TFA) in water and 1 µl was applied to target plate (Bruker, USA). The sample was allowed to dry at room temperature for 15–20 min. Spectrum was obtained by striking 3000 shots in an acquisition mass range of 70,000–100,000 Da. Final spectrum was subjected to smoothing, baseline subtraction and centroiding.

Isoelectric focusing was done for determination of isoelectric point of *Pca*-Pol. SERVA precast IPG strip of linear pH gradient from pH 4 to 7 was used. Electrophoresis was performed on 2D-IEF-SYS (Scie-Plas, UK).

Assay for DNA polymerase activity DNA polymerase activity was monitored by measuring incorporation of TTP [methyl-^{3}H] in activated calf thymus DNA. The reaction mixture, in 20 µl, contained: 25 mM Tris–Cl pH 8.5, 4 mM $MgCl_2$, 100 µM each of dATP, dGTP, dCTP, dTTP, 0.5 µCi TTP [methyl-^{3}H] (85 Ci/mmol), 5 µg activated calf thymus DNA, 0.2 mg/ml BSA and 0.1% Tween 20. The mixture was preincubated at 75°C for 5 min before the addition of the enzyme. After the addition of the enzyme, aliquots were removed from the reaction mixture at various time intervals and spotted onto DE-81 filter paper discs (23 mm diameter, Whatman, Madison, UK). The discs were dried, washed three times in sodium phosphate buffer pH 7.0 followed by washing in 70% ethanol (2 min for each wash) and dried. The incorporated radioactivity on the dried filter discs was measured in counts per second (cps) by using Raytest Malisa scintillation counter (Berlin, Germany). One unit of DNA polymerase was defined as the amount of the enzyme required to incorporate 10 nmol [^{3}H]TTP into a polynucleotide fraction (adsorbed on DE-81 filter disc) at 75°C in 30 min.

The effect of concentration of dNTPs on the polymerase activity was analyzed by using biotin labeled primer-template. A 40-mer primer (5′-biotin-CGCACCGTGACGCCAAGCTTGCATTCCTACAGGTCGACTC-3′) annealed to an 80-mer template (5′-biotin-CGTTGCTGACAAACGGGCCGGTCAACAATCCTCTGGAGTCGACCTGTAGGAATGCAAGCTTGGCGTCACGGTGCGCCAAC-3′) was incubated with 0.2U *Pca*-Pol for 5 min at 75°C either in the absence or presence of various concentrations of dNTPs in buffer containing 25 mM Tris–Cl pH 8.5, 4 mM $MgCl_2$, 0.2 mg/ml BSA and 0.1% Tween 20. Reactions were stopped by addition of formamide buffer and incubation on ice. DNA was resolved on 15% polyacrylamide gel in the presence of 7 M urea. After transfer of DNA to Hybond N+ membrane (GE Healthcare), formation of 75-mer product was detected by using biotin chromogenic detection kit (Fermentas).

Effect of inhibitors The effect of aphidicolin and dideoxythymidine triphosphate (ddTTP) on the activity of *Pca*-Pol was investigated. Enzyme activity was measured as described above except for analyzing the effect of ddTTP where the reaction mixture did not contain dTTP. In addition to other components, the reaction mixtures contained the indicated concentration of the inhibitor. Assay was performed in the presence of either aphidicolin (0–1000 µM) or ddTTP (ddTTP:TTP [methyl-^{3}H] 0–10). In order to investigate the effect of ddTTP, the concentration of TTP [methyl-^{3}H] was kept constant in all the reaction mixtures while that of ddTTP was varied to achieve a required concentration ratio.

Assay for exonuclease activity *Pca*-Pol was also analyzed for the presence of associated 3′→5′ exonuclease activity. In order to prepare 3′ labeled DNA substrate, *Hin*dIII digested λ phage DNA was filled in by exonuclease deficient Klenow fragment in the presence of 100 µM each of dATP, dGTP, dCTP and 1 µCi TTP [methyl-^{3}H]. After labeling, the substrate DNA was purified by sephadex G 25 spin column (GE healthcare). 3′→5′ exonuclease activity was assayed in a 50 µl reaction mixture containing 25 mM Tris–Cl pH 8.5, 4 mM $MgCl_2$ and the labeled substrate DNA. The reaction mixture was preincubated at 75°C for 5 min before the addition of the enzyme. After the addition of the enzyme, aliquots were removed from the reaction mixture at various time intervals and spotted onto DE-81 filter paper discs. The discs were washed and radioactivity was measured as described above. Exonuclease activity was monitored by measuring decrease in radioactivity bound to filter disc. This value was used to calculate the percentage of radioactivity released from labeled substrate DNA.

Analysis of 3′→5′ exonuclease activity was also done by monitoring the degradation of a 40-mer primer annealed to an 80-mer template (described above). The annealed primer-template was incubated with 0.5U of either *Pca*-Pol or *Pfu* or *Taq* DNA polymerases at 75°C for 1 h. For single strand dependent exonuclease assay, only 40-mer was used. After incubation, DNA was resolved on 15% polyacrylamide gel in the presence of 7 M urea, transferred to Hybond N+ membrane (GE Healthcare) and detected by using biotin chromogenic detection kit (Fermentas).

PCR with purified *Pca*-Pol To evaluate the extent of amplification produced by the enzyme, PCR amplification of DNA fragments of various lengths was performed. DNA fragments cloned in either pTZ57R/T or pET-22b were used as templates in PCR. Various lengths of amplified products were obtained by using different sets of specific primers. In addition to the respective primer-template set, the PCR mixture in 20 µl contained 10 mM Tris–Cl pH 8.5, 4 mM $MgCl_2$, 200 µM each dNTP, 0.2 mg/ml BSA and 2.5 U *Pca*-Pol. PCR conditions were: initial denaturation at 95°C for 2 min; followed by 30 cycles of: denaturation at 95°C for 30 s, annealing at 52°C for 30 s, extension at 72°C (45 seconds per kb); and final extension at 72°C for 10 min. The entire sample, after PCR, was analyzed by agarose gel electrophoresis.

RESULTS

Gene cloning and sequence analysis A 2352 bp *Pca*-pol open reading frame (ORF) coding for 783 amino acid protein was cloned in pTZ57R/T followed by cloning in expression vector pET-21a(+). Restriction site of *Nde*I was introduced in the forward primer Pol2N which changed the start codon GTG (coding for valine) to ATG (coding for methionine). After cloning, gene sequence was determined and deduced amino acid sequence of the encoded protein was elucidated.

The deduced amino acid sequence of the *Pca*-Pol was aligned and compared to those of other archaeal family B DNA polymerases (Fig. S1). Amino acid sequence alignment revealed the presence of conserved 3′→5′ exonuclease motifs (19) and 5′→3′ polymerase motifs (5). In pairwise alignment, *Pca*-Pol showed 78% identity to *Pyrobaculum islandicum* DNA polymerase (accession no. AF195019), 54% to *Pyrodictium occultum* DNA polymerase (PolB accession no. D38574), 51% to *Aeropyrum pernix* DNA polymerase (Pol II accession no. AB017501), 35% to *Pyrococcus furiosus* DNA polymerase (accession no. D12983) and 30% to *Sulfolobus solfataricus* DNA polymerase (accession no. Y08257).

Production and purification of *Pca*-Pol *E. coli* BL21 CodonPlus (DE3)-RIL cells harboring pol-pET21a expression vector were grown in LB medium and gene expression was induced with IPTG. The cells