

**Recombinant His-tagged DNA polymerase.  
I. Cloning, purification and partial characterization  
of *Thermus thermophilus* recombinant DNA polymerase<sup>Ⓞ</sup>**

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Received: 15 April, 1998; revised: 18 June, 1998

**Key words:** PCR, RT-PCR, DNA polymerase, reverse transcriptase, *Thermus thermophilus*, recombinant protein

The *Tth* DNA polymerase gene from the thermophilic *Thermus thermophilus* (strain HB8) was amplified, cloned and expressed in *Escherichia coli*. The recombinant DNA polymerase containing a polyhistidine tag at the N-terminus was isolated in a single step by Ni<sup>2+</sup> affinity chromatography. The purified recombinant enzyme, showing high polymerase activity contained 43 additional amino-acid residues (including a cluster of six histidine residues inserted for purification of the recombinant protein by metal-affinity chromatography) at N-terminus. The applied overexpression system was very efficient giving 700000 u of DNA polymerase activity from 1 liter of induced culture. The enzyme was characterized and displayed high DNA polymerase and reverse transcriptase activities and high thermostability as compared to the native *Tth* DNA polymerase.

Beginning with the discovery and characterization of DNA polymerase I from *Escherichia coli* by Arthur Kornberg and colleagues in 1950, a variety of DNA polymerases have been isolated from prokaryotic and eukaryotic sources. Study of these enzymes has provided key insights into nucleic acid metabolism. Additionally, DNA polymerases have been exploited in specialized molecular biology techniques. For example, DNA polymerase I has

been the enzyme of choice for nick translation of DNA. Both the thermostable *Taq* DNA polymerase [1, 2] and a modified version of T7 DNA polymerase [3] have been used for dideoxy sequencing of DNAs with retained secondary structure [3, 4]. Heat-stable polymerases such as *Taq* DNA polymerase or *Tth* DNA polymerase have also been key elements in the development of the polymerase chain reaction technique [5]. A number of thermophilic DNA

<sup>Ⓞ</sup>The work was supported by Technical University of Gdańsk and DNA-Gdańsk.

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**Abbreviations:** IMAC, immobilized ion affinity chromatography; NTA, nitrilotriacetic acid; IPTG, isopropylthiogalactosidase; LB, Luria-Bertani broth.

polymerases have been isolated previously and characterized from both mesophilic eubacteria and archaea sources. Those that have been analyzed are monomeric in solution with molecular masses of 80–115 kDa [6–8]. As expected, these enzymes have elevated temperature optima and thermal stabilities that roughly correspond to the thermal extremes of the environment from which they were isolated. Despite the fact that thermal stabilities of the native proteins vary from enzyme to the enzyme, an optimal temperature for polymerization of 70–80°C is common. As it has been pointed out by many authors, this suggests that the template stability rather than the intrinsic enzyme stability determines the optimal temperature for polymerization [7, 8]. Due to their thermostability, the structure and function relationships and the potential industrial applications of many thermostable enzymes such as DNA polymerases are of considerable interest to researchers. However, most native thermostable enzymes are synthesized by the thermophilic bacteria at a very low level and therefore their purification is cumbersome. More than 50 DNA polymerase genes have been cloned from various organisms, including thermophiles and archaea, and sequenced. Amino-acid sequences deduced from their nucleotide sequences can be classified into four major groups: *Escherichia coli* DNA polymerase I (family A), DNA polymerase II (family B,  $\alpha$ -like DNA polymerases), DNA polymerase III (family C), and others (family X) [9].

In 1975 a new concept for affinity purification of proteins was presented by Porath and co-workers [10]. Their method is based on the interaction between the side chains of certain surface amino acids, particularly histidines, and immobilized transition metal ions. The method is known as an immobilized metal ion affinity chromatography (IMAC). The metal ions are immobilized by the use of a chelating agent capable of transferring them to proteins. Several gene fusion systems employing histidine-rich tags for purification of recombi-

nant proteins have been described so far. The tags, either N- or C-terminal, consisting of consecutive histidine residues binding selectively to immobilized  $\text{Ni}^{2+}$  ions, were described by Hochuli and co-workers [11]. The adsorption of the poly-His-tagged proteins to a metal-chelate adsorbent was performed at neutral or slightly alkaline pH, at which the imidazole group of histidine is not protonated [12]. The expressed fusion proteins were recovered with the purity above 90% in a single step using  $\text{Ni}^{2+}$ -nitrilotriacetic acid (NTA) as a metal chelate adsorbent and elution at low pH, or in a competition reaction using imidazole [11, 12]. The usefulness of polyhistidines as a fusion tag for protein purification has been demonstrated for recombinant fusion proteins produced in a wide range of host cells including *E. coli*, *Saccharomyces cerevisiae*, mammalian cells, and baculovirus-infected *Spodoptera frugiperda* insect cells [13–18]. An important advantage of the His<sub>6</sub> affinity tag is the possibility of purifying proteins under denaturing conditions, thus facilitating purification of less soluble proteins.

The results of this study and other published earlier demonstrate that thermostable enzymes can be efficiently synthesized and produced in a biologically active form in the over-expression system of mesophilic bacteria.

## MATERIALS AND METHODS

**Bacterial strains, plasmids, enzymes and reagents.** *Thermus thermophilus* HB-8 (DSM 579, DSMZ-Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH, Braunschweig, Germany) was used as a source of total DNA. DH5 $\alpha$  *E. coli* strain was used for preparation of plasmids and BL21 (DE3) pLysS (Promega, U.S.A.) *E. coli* strain carrying T7 RNA polymerase gene under the control of a chromosomal *lacUV5* gene [19] was used for expression of *Tth* DNA polymerase. The plasmid pET30-LIC (Novagen, U.K.) was used for the construction of expres-

sion system. The *E. coli* cells with inserted plasmids were cultured aerobically at 37°C in LB medium supplemented with 34 µg/ml kanamycin for DH5α strain or with 34 µg/ml kanamycin and 50 µg/ml chloramphenicol for BL21 (DE3) pLysS strain. Restriction, modification enzymes and native *Tth* DNA polymerase were purchased from Promega and entokinase from Novagen (U.K.). *Pwo* DNA polymerase (Delta2, DNA-Gdańsk), other reagents for the PCR and Ni<sup>2+</sup>-TED Sepharose columns were obtained from DNA-Gdańsk (Poland). All other reagents were purchased from Sigma.

**Assays for relative DNA polymerase and reverse transcriptase activity.** The relative DNA polymerase activity was determined by comparing the PCR results obtained with His<sub>6</sub>-tagged *Tth* DNA polymerase with those for native *Tth* DNA polymerase from Promega. The PCR system for specific detection of BLV (Bovine Leukemia Virus) was applied to this assay. The PCR conditions and primers were as described by Kubiś *et al.* [20] with some modifications. The primers: ZM4-5' CTC GCC CTC CCG GAC GCC CA and ZM5-5' GCT AGG CCT AAG GTC AGG GCC GC were applied to amplify a 218 bp fragment of virus *env* gene. Plasmid pENV1 was used as a matrix DNA. A sample of 1 µl of plasmid DNA (10 pg) was combined with 2.5 µl of 10 × reaction buffer (500 mM KCl, 100 mM Tris/HCl, pH 8.8, 20 mM MgCl<sub>2</sub>, 1% Triton X-100), 2.5 µl of a deoxynucleoside triphosphates (dNTPs) mixture (2.5 mM of each dNTP), 1 µl of each primer (10 µM) and 16 µl of water. This mixture was supplemented with 1 µl of recombinant enzyme fraction or 1 u of the purified (final preparation) His<sub>6</sub>-tagged *Tth* DNA polymerase or 1 u of the native *Tth* DNA polymerase. Amplification reactions were performed with the use of an automated thermocycler (thermocycler Hot-Shot 25, DNA-Gdańsk) according to the following scheme: an initial cycle of 1 min at 94°C was followed by 30 cycles of denaturation for 30 s at 94°C; annealing and elongation of primers for 1 min

at 64°C; and one extension step at 72°C for 5 min.

PCR specific products were separated by electrophoresis on a 2% agarose gel using 1 × Tris/borate/EDTA running buffer at a field strength of 8 V/cm. The DNA was visualised on an ultraviolet transilluminator following ethidium bromide staining, and photographed. The relative amounts of PCR products were estimated using the BioDoc (Biometra, Germany) densitometry system.

The RNA for examination of the reverse transcriptase activity of the His<sub>6</sub>-tagged *Tth* DNA polymerase was isolated from HeLa cells culture using RNA Prep Plus Kit (A & A Biotechnology, Poland). The primer that anneals to the human beta-2-microglobulin gene, MicG12: 5'-ATC TTC AAA CCT CCA TGA TG was used as primer for cDNA synthesis (RT primer). The MicG11 (5'-ACC CCC ACT GAA AAA GAT GA) and MicG12 primers were used for PCR amplification generating a 114 bp amplicon. The RT mixture (20 µl) contained 10 µM Tris/HCl (pH 8.3), 90 mM KCl, 0.2 mM each of deoxynucleoside triphosphates (dATP, dCTP, dGTP, dTTP), 1 mM MnCl<sub>2</sub>, 0.2 µM MicG12 primer, 1 u of His<sub>6</sub>-tagged *Tth* DNA polymerase or 1 u of native *Tth* DNA polymerase and 1 µg of total RNA. Reactions were run at 94°C for 2 min, then at 55°C for 2 min (annealing of RT primer) and at 70°C for 20 min for the RT step. Following the RT reaction, 30 µl of PCR mixture was added, containing 10 mM Tris/HCl (pH 8.3), 0.1 mM KCl, 0.75 mM EGTA, 0.05% Tween-20, 5% glycerol, 0.2 mM each of dNTPs, 2.5 mM MgCl<sub>2</sub> and 0.2 µM of MicG11 primer. The incubation mixture (50 µl) was then subjected to amplification as follows: 30 s at 93°C, 30 s at 55°C, and 30 s at 72°C for 35 cycles. Aliquots of 10 µl were analyzed by electrophoresis on a 6% polyacrylamide gel.

**Examination of thermostability of the His<sub>6</sub>-tagged *Tth* DNA polymerase.** For the thermostability studies His<sub>6</sub>-tagged *Tth* DNA polymerase and native *Tth* DNA polymerase were heated at 95°C for 5, 15 and 30 min prior

to the PCR reaction. After various incubation periods enzyme samples were withdrawn and tested for DNA polymerase activity using specific PCR amplification with pENV1 matrix as described above. The relative amounts of PCR products were estimated using the BioDoc (Biometra, Germany) densitometry system.

## RESULTS AND DISCUSSION

### Cloning of the *Thermus thermophilus* *polA* gene and construction of the recombinant vector producing His<sub>6</sub>-tagged *Tth* DNA polymerase

Construction of a high level expression plasmid for the *T. thermophilus* *polA* gene in *E. coli* was achieved using the pET30-LIC expression vector. Oligonucleotide primers (forward: OR-LIC1 5'-GAC GAC GAC AAG ATG GAG GCG ATG CTT CCG CTC, 33 nt and reverse: OR-LIC2 5'-GAG GAG AAG CCC GGT CCC CTT GAA CTT CCT CTA AAC GGC, 39 nt) used for PCR amplification were designed on the basis of the *T. thermophilus* *polA* gene nucleotide sequence (GenBank<sup>TM</sup>/EMBL accession number: D28878). In 50 µl of reaction mixture,

200 ng of *T. thermophilus* total DNA and 0.2 µmole of each primer were used. The mixture was incubated at 94°C for 2 min, then subjected to 30 cycles of 30 s of denaturation at 94°C, 1 min of annealing at 62°C, and 3 min of elongation at 72°C. After 30 cycles the reaction mixture was incubated at 72°C for 5 min and then cooled to +4°C. The amplification product was analyzed by electrophoresis on 1% agarose gel. The amplification product corresponding to the full-size gene (approximately 2500 bp) was treated with T4 DNA polymerase in the presence of dATP and inserted into pET30-LIC vector, to obtain the recombinant pTth1 plasmid.

The nucleotide sequences of that construct were confirmed by sequencing analysis to ensure that the reading frame was correct. The primer sequences, nucleotide and amino-acids sequences of the pTth1 plasmid near the site of the *Tth* DNA polymerase gene insertion are shown in Fig. 1. The obtained genetic construct retained the open reading frame, and the target protein contained 43 additional amino-acid residues at N-terminus, including a cluster of six histidine residues inserted for purification of the recombinant protein by metal-affinity chromatography.



Figure 1. The sequences of primers, nucleotides and amino acids of the pTth1 plasmid close to the site of the DNA polymerase gene insertion.

### Expression of the recombinant His<sub>6</sub>-tagged *Tth* DNA polymerase

The recombinant plasmid pTth1 was transformed into *E. coli* BL21 (DE3) pLysS. Bacterial colonies grown on chloramphenicol and kanamycin-containing agar were assayed for DNA polymerase activity using specific PCR amplification with pENV1 matrix. The clones displaying high DNA polymerase activity were selected and grown separately in LB medium supplemented with chloramphenicol and kanamycin. Overexpression was induced by addition of IPTG (final concentration, 1 mM) at various stages of culture ( $A_{660}$ : 0.1, 0.2, 0.3, 0.4, 0.5 and 0.6). The best induction of His<sub>6</sub>-tagged *Tth* DNA polymerase synthesis was obtained at  $A_{660}$  of 0.3. At  $A_{660} > 0.4$  the synthesis of the recombinant protein and DNA polymerase activity were low.

### Purification of recombinant His<sub>6</sub>-tagged *Tth* DNA polymerase

*E. coli* strain BL21 (DE3) pLysS transformed with pTth1 were grown at 37°C in 400 ml of LB containing 50 µg/ml chloramphenicol and 34 µg/ml kanamycin to an  $A_{660}$  of 0.3. IPTG was then added to the final concentration of 1 mM. The cells were harvested after 4 h by centrifugation (3 g of cell mass) and the pellet was resuspended in 10 ml of buffer B (20 mM Tris, pH 7.9, 500 mM NaCl, 5 mM imidazole, 0.1% Triton X-100). The cells were disrupted by sonication, then the lysate was heated at 75°C for 30 min and centrifuged at 16000 × g for 20 min. The supernatant (about 9 ml) was then applied to a

Ni<sup>2+</sup>-TED Sepharose column (10 ml of bed volume) equilibrated with four volumes of buffer B. Next, the column was washed several times with 10 ml portions of the same buffer. His<sub>6</sub>-tagged *Tth* DNA polymerase was eluted twice with 10 ml of washing buffer B containing 60 mM imidazole (buffer W) and once with 5 ml of buffer B containing 1 M imidazole (buffer E). The eluted fractions were pooled and dialyzed against a buffer containing 20 mM Tris pH 7.9, 100 mM KCl, 0.1% Triton X-100, 0.1 mM EDTA, 1 mM dithiothreitol, and 50% glycerol. The native *Tth* DNA polymerase enzyme was obtained by proteolytic cleavage of 3 µg of His<sub>6</sub>-tagged *Tth* DNA polymerase with 0.05 u of enterokinase in a buffer containing 20 mM Tris/HCl, pH 7.4, 50 mM NaCl and 2 mM CaCl<sub>2</sub> at 23°C for 16 h.

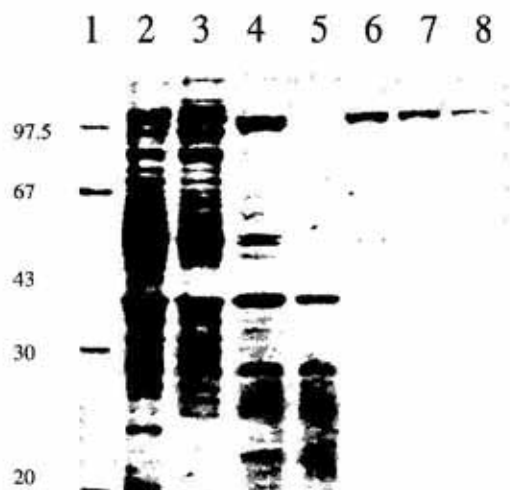
The concentration of purified enzymes was determined from ultraviolet absorbance, using the extinction coefficient for  $A_{280} = 0.78$  for 1 mg/ml, calculated from the number of Trp and Tyr residues in the sequence (using the Protean program of DNASTar, Madison, WI, U.S.A.) and also estimated by the PIERCE BCA assay using BSA as a standard. The results of the purification procedure are presented in Table 1.

Purity of the enzyme was examined by sodium dodecyl sulfate gel electrophoresis (PAGE) (Fig. 2) and the activity of the enzyme during the purification procedure was determined by PCR specific reaction (Fig. 3). Before the chromatography step the cells lysate was heat treated at 75°C for 30 min. The heat denaturation step, which takes advantage of *Tth* DNA polymerase thermostability result-

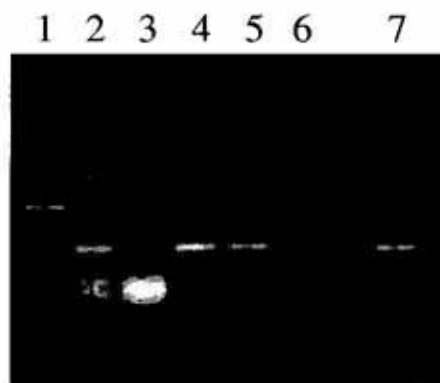
Table 1. Purification of the His<sub>6</sub>-tagged *Tth* DNA polymerase

Enzyme fraction	Step of purification	Total protein (mg)	Specific activity (units/mg)	Recovery (%)	Purification fold
I	Crude supernatant	150	ND	ND	ND
II	Clarified lysate after heat treatment	29	10000	100	1
III	Eluate from metal affinity chromatography	14	20000	97	2

ND, not determined.



ing in precipitation of the vast majority of contaminating cellular proteins, allows to obtain on polyacrylamide gel, in approximately 60% yield (Table 1), a single species band of about 98 kDa which corresponds to fusion His<sub>6</sub>-tagged *Tth* DNA polymerase (Fig. 3, lane 4). For the final purification, the clarified medium was chromatographed on a Ni<sup>2+</sup>-TED Sepharose column. The recombinant enzyme was eluted from the column as a single peak in about 97% purity at the same position as the



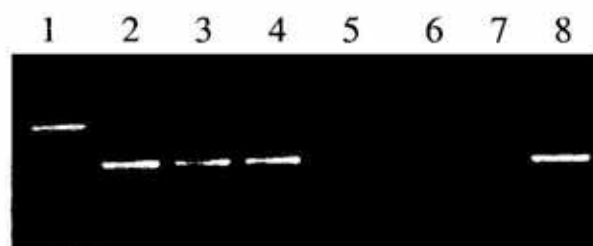
**Figure 3.** Monitoring of the His<sub>6</sub>-tagged *Tth* DNA polymerase activity by PCR specific reaction during the purification procedure.

Lane 1, the molecular mass marker (501, 489, 404, 331, 242, 190, 147, 111, 110 bp); lane 2, clarified supernatant after heat treatment; lane 3, flow through fraction obtained with buffer B; lane 4, the first fraction eluted with buffer W (60 mM imidazole); lane 5, second fraction eluted with buffer W (60 mM imidazole); lane 6, fraction eluted with buffer E (1 M imidazole); lane 7, *Tth* DNA polymerase (1 u, Promega).

**Figure 2.** SDS-electrophoresis in 10% polyacrylamide gel of the fractions obtained by purification by affinity chromatography on the Ni<sup>2+</sup>-TED Sepharose column of the recombinant His<sub>6</sub>-tagged *Tth* DNA polymerase from *E. coli* BL21 (DE3) pLysS containing plasmid pTth1.

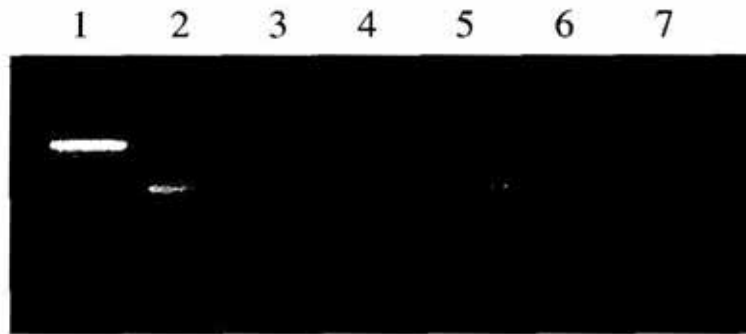
Lane 1, low molecular mass marker (Pharmacia LKB); lane 2, lysate from *E. coli* BL21 (DE3) pLysS; lane 3, lysate from *E. coli* BL21 (DE3) pLysS + pTth1; lane 4, cleared lysate after heat treatment; lane 5, flow through fraction with buffer B; lane 6, first fraction eluted with buffer W; lane 7, second fraction eluted with buffer W; lane 8, fraction eluted with buffer E.

band of fusion His<sub>6</sub>-tagged *Tth* DNA polymerase after heat treatment (Fig. 2, lanes 6–8). A band corresponding to a 98 kDa protein was observed on SDS/PAGE of crude extracts of *E. coli* BL21 (DE3) pLysS + pTth1 cultures after IPTG induction (Fig. 2, lane 3). This band was absent in the control crude extracts of *E. coli* BL21 (DE3) pLysS cultures (Fig. 2, lane 2). A pronounced enrichment in the 98 kDa polypeptide depicted in Fig. 2 (lane 4) after the heat denaturation step (compare lane 4 with lane 3 in Fig. 2) should be noted. After dialysis of recombinant protein against the storage buffer, the activity of the purified His<sub>6</sub>-tagged *Tth* DNA polymerase (25 ml) isolated from 400 ml of the *E. coli* culture was



**Figure 4.** DNA polymerase activity of the enzyme fraction after purification by affinity chromatography on the Ni<sup>2+</sup>-TED Sepharose column of the His<sub>6</sub>-tagged *Tth* DNA polymerase by using specific PCR amplification.

Lane 1, the molecular mass marker (501, 489, 404, 331, 242, 190, 147, 111, 110 bp); lane 2, 1 µl of enzyme fraction; lanes 3–7, enzyme fraction diluted: 1/2, 1/4, 1/8, 1/16 and 1/32, respectively; lane 8, 1 µl (1 u) of native *Tth* DNA polymerase (Promega).



**Figure 5.** Comparison of thermostability of the recombinant His<sub>6</sub>-tagged *Tth* DNA polymerase (lanes 2–4) and native *Tth* DNA polymerase (Promega) (lanes 5–7) using specific PCR amplification.

The enzymes were heated at 95°C before the PCR reaction for 5 min (lanes 2 and 5), 15 min (lanes 3 and 6) and 30 min (lanes 4 and 7). Lane 1 represents the molecular mass marker (501, 489, 404, 331, 242, 190, 147, 111, 110 bp).

11.2 u/μl (i.e., 700000 u/l medium) (see also Table 1)

#### The relative DNA polymerase and reverse transcriptase activity

As shown in Fig. 4, the polymerases from the recombinant (His<sub>6</sub>-tagged *Tth* DNA polymerase) and native sources showed only minor differences in DNA polymerase activity. The relative His<sub>6</sub>-tagged *Tth* DNA polymerase activity was also comparable to that of the His<sub>6</sub>-tagged *Tth* DNA polymerase cleaved with enterokinase (not shown) using the same specific PCR amplification test.

The reverse transcriptase activity of the purified His<sub>6</sub>-tagged *Tth* DNA polymerase was analyzed using RNA from HeLa cells with the primers corresponding to the human beta-2-microglobulin gene. The reverse transcriptase activity of the native and the recombinant (His<sub>6</sub>-tagged *Tth* DNA polymerase) enzyme (not shown) was the same.

#### Thermostability

His<sub>6</sub>-tagged *Tth* DNA polymerase was stable and active at high temperature. The thermal inactivation level at 95°C of the purified enzyme and the native *Tth* DNA polymerase were compared as shown in Fig. 5.

The presented system of His<sub>6</sub>-tagged *Tth* DNA polymerase cloning and overexpression shows some advantages over, and differ-

ences with, the similar system described by Smirnow and co-workers [21]: (i) more efficient overexpression vector (pET30-LIC with *lac* operator), (ii) longer additional N-terminus (43 aa in relation to 12 aa) having no effect on the polymerase activity, (iii) 5 times higher productivity and activity from 1 liter of induced culture (700000 u in relation to 130000 u).

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