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QUARTERLY

Recombinant His-tagged DNA polymerase. II. Cloning and purification of *Thermus aquaticus* recombinant DNA polymerase (Stoffel fragment)[o]

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The Stoffel DNA fragment, shortened by 12 bp from 5′ end, coding for Stoffel DNA polymerase (missing 4 amino acids at N-terminus of Stoffel amino-acids sequence) from the thermophilic *Thermus aquaticus* (strain YT-1) was amplified, cloned and expressed in *Escherichia coli*. The recombinant Stoffel fragment contained a polyhistidine tag at the N-terminus (21 additional amino acids) that allowed its single-step isolation by Ni^{2+} affinity chromatography. The enzyme was characterized and displayed high DNA polymerase activity and thermostability evidently higher than the native Taq DNA polymerase.

DNA polymerase from *Thermus aquaticus* (*Taq* DNA polymerase) is an enzyme that is of very considerable biological, technological, and economic importance [1–3]. Like DNA polymerases from other organisms, it is the central participant in the replication of genetic information with great fidelity; unlike similar enzymes from mesophilic organisms, it carries out this function at elevated temperatures [4]. It is also widely used in the polymerase chain reaction (PCR) experiments [5]. PCR occupies a central role in the technology of molecular biology and has over the past few years become an indispensable tool in the laboratory [6]. According to classifications of DNA polymerases based on sequence similarities, *Taq* polymerase is a member of the group of other bacterial DNA polymerases including DNA polymerase I from *Escherichia coli* [7–9]. As predicted from the DNA sequence, the protein chain of *Taq* consists of 832 amino acids, and a molecular mass of 93925 Da [10]. *Taq* DNA polymerase has neither measurable 3′-5′ exonuclease activity nor any amino-acid ho-

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Abbreviations: IPTG, isopropylthiogalactoside; LB, Luria-Bertani broth; NTA, nitrilotriacetic acid; *Taq* DNA polymerase, DNA polymerase from *Thermus aquaticus*.

mology in the region of other DNA polymerases showing 3′-5′ exonuclease activity [10]. In contrast, *Taq* has 5′-3′ exonuclease activity and its N-terminal region (residues 1–300) shows extensive amino-acid similarity to the putative 5′-3′ exonuclease region of *E. coli* Pol I and several other Pol I family members and related phage exonucleases [10–12]. Deletion of the 289 N-terminal amino acids of the native *Taq* polymerase preparation (832 amino acids) results in a 61 kDa active fragment, the so-called Stoffel fragment, that lacks any intrinsic 5′-3′ exonuclease activity (< 0.00001 pmol/s per pmol enzyme), but its thermostability is approximately twice as high as that of the native *Taq* DNA polymerase (half-life at 97.5°C of 20 min and 10 min, respectively) [10]. Another important advantage of the Stoffel fragment over the native *Taq* polymerase is a broader range of magnesium ion concentrations (2–10 mM) optimal for the polymerase activity. The lack of 5′-3′ exonuclease activity improves the amplification of circular templates (e.g., plasmid DNA). The higher thermostability allows to apply higher denaturation temperatures in PCR cycles, which is particularly important for the efficient amplification of fragments with the high proportions of GC and complex secondary structures. The higher range of magnesium concentrations over which the Stoffel fragment is active might contribute toward improving PCR methods and it might also prove useful in the simultaneous amplification of different fragments in a single tube (multiplex PCR).

More than 50 DNA polymerase genes have been cloned from various organisms, including thermophiles and archaea, and sequenced. The tags, either N- or C-terminal, consisting of consecutive histidine residues binding selectively to immobilized Ni^{2+} ions, were used by Hochuli *et al.* [13] for purification of recombinant proteins. The expressed fusion proteins were recovered with the purity of more than 90 in a single step using a Ni^{2+}-nitrilotriacetic acid (NTA) adsorbent and elution at low pH, or by competition using imidazole [13, 14]. The use of polyhistidines as fusion tags for protein purification has been demonstrated for recombinant fusion proteins produced in a wide range of host cells including *E. coli*. Two thermostable DNA polymerases, *Taq* and *Tth*, were also cloned and overexpressed in *E. coli* as fusion His_6-tagged proteins [15]. Cloning and expression of these His_6-tagged polymerases allows to perform rapid, simple purification of large quantities of research quality enzymes in a single chromatography step.

The results of this study demonstrate that His_6-tagged Stoffel fragment of *Taq* DNA polymerase can be efficiently synthesized in a biologically active form in the *E. coli* overexpression system and large amounts of active enzyme can be purified using a single-step procedure.

MATERIALS AND METHODS

Bacterial strains, plasmids, enzymes and reagents. *Thermus aquaticus* YT-1 was used as a source of total DNA. DH5α *E. coli* strain was used for preparation of plasmids and cloning, and BL21 (DE3) pLysS (Promega, U.S.A.) was applied to expression of Stoffel fragment of *Taq* DNA polymerase [16]. The plasmid pET15b (Novagen, U.K.) was used for construction of the expression system. The *E. coli* cells with plasmids were cultured aerobically at 37°C in LB medium supplemented with 100 μg/ml ampicillin for DH5α strain or with 100 μg/ml ampicillin and 50 μg/ml chloramphenicol for BL21 (DE3) pLysS strain. Restriction, modification enzymes were purchased from Promega and expression vector pET15b from Novagen (U.K.). *Pwo* DNA polymerase (Delta2), other reagents for PCR and Ni^{2+}-TED Sepharose columns were obtained from DNA-Gdańsk (Poland). Other reagents for protein purification were purchased from Sigma.

Assays for relative DNA polymerase activity. The relative DNA polymerase activity was determined for comparative purpose as described in part I using the native *Taq* DNA polymerase from Promega for comparison and PCR assay for specific detection of BLV (Bovine Leukemia Virus) [17].

The examination of His_6-tagged Stoffel fragment thermostability. The studies of thermostability of the His_6-tagged Stoffel fragment were performed as described in part I with native *Taq* DNA polymerase (Promega) for comparison.

RESULTS AND DISCUSSION

Cloning of the Stoffel fragment nucleotide sequence and construction of the recombinant vector producing His_6-tagged Stoffel fragment

The PCR amplification of Stoffel fragment nucleotide sequence was carried out with oligonucleotide primers (forward: HS1 5′-CGA ATT CAT ATG **GCC CTG GAG GAG GCC CCC**, 30 nt, containing *Eco*RI and *Nde*I recognition sites; reverse: HS2 5′-GAA GCT TAC **TCC TTG GCG GAG AGC CA**, 26 nt, containing a *Hin*dIII recognition site). The primers used for the PCR amplification were designed on the basis of the *T. aquaticus polA* gene nucleotide sequence (GenBank™/EMBL accession number: J04639). The bolded 3′ nucleotide sequences of the primers which are complementary to appropriate nucleotide sequences of the *polA* gene and 5′ ends of the primers containing recognition sites for restriction endonucleases, have been designed to facilitate cloning. In 50 μl of reaction mixture 200 ng of *T. aquaticus* total DNA and 0.2 μM 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 60°C, and 2 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 a 1% agarose gel. The amplification product (5 μg), corresponding to the Stoffel DNA fragment (1640 bp), digested with *Eco*RI and *Hin*dIII, was isolated from an agarose gel band using Gel-Out kit (A & A Biotechnology,

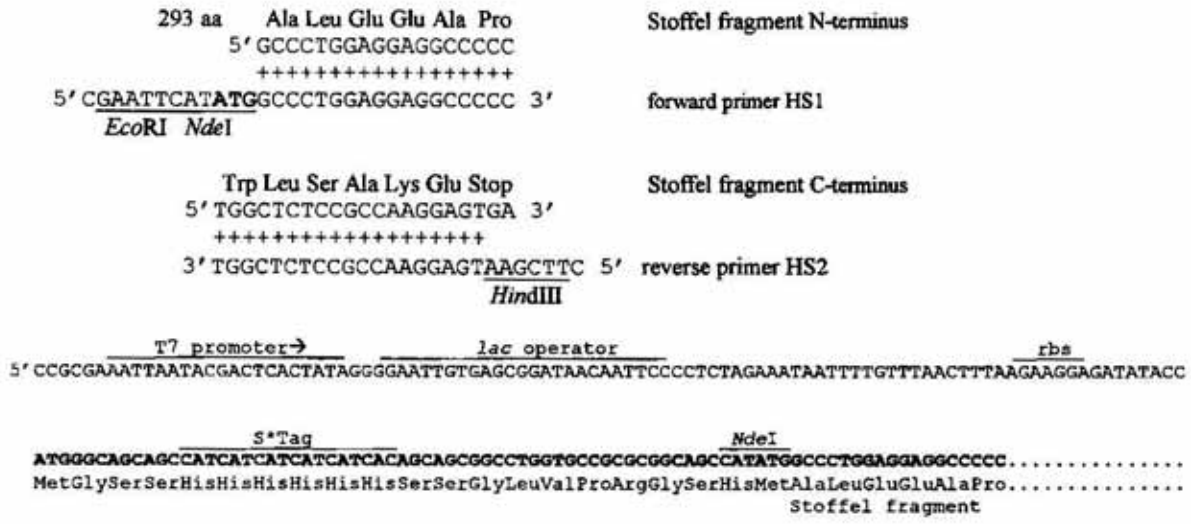

Figure 1. The sequences of primers, nucleotides and amino acids of the pHisStoff1 plasmid near the site of the Stoffel DNA fragment insertion.

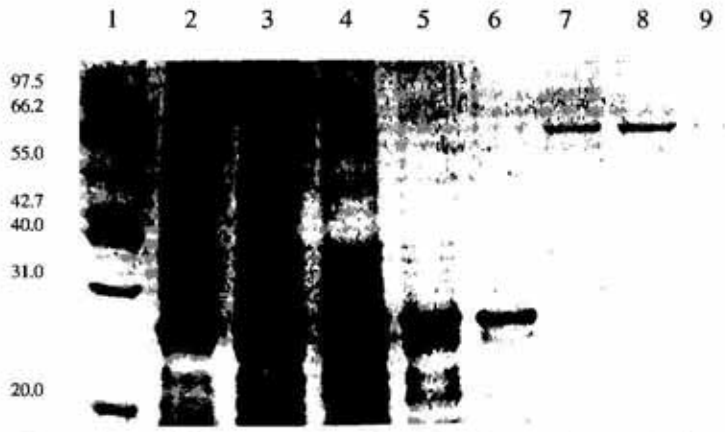

Figure 2. SDS-electrophoresis in 10% polyacrylamide gel of the fractions obtained by purification of the recombinant His_6-tagged Stoffel fragment from *E. coli* BL21 (DE3) pLysS containing plasmid pHisStoff1, by metal-affinity chromatography on Ni^{2+}-TED Sepharose.

Lane 1, mid-range molecular mass markers (Promega); lane 2, lysate from *E. coli* BL21(DE3) pLysS; lane 3, lysate from *E. coli* BL21 (DE3) pLysS + pHisStoff1; lane 4, cleared lysate after heat treatment; lane 5, flow through fraction obtained with buffer B; lane 6, fraction washed out with buffer B20; lane 7, fraction eluted with buffer B40; lane 8, fraction eluted with buffer B60; lane 9, fraction eluted with buffer B100.

Poland). The purified fragment was ligated into pUC19 *Eco*RI–*Hin*dIII sites. *E. coli* DH5α cells were transformed with the ligation mixture giving over twenty white colonies. Ten such colonies were assayed for the presence of the Stoffel DNA fragment by restriction analysis. Two selected clones were verified by sequencing using the dideoxy chain termination method. The isolated recombinant plasmid, pUC19Stoff, after digestion with *Nde*I and *Hin*dIII restriction enzymes was used as a source of Stoffel DNA fragment (1638 bp) to be cloned into the pET15b *Nde*I–*Hin*dIII sites giving the recombinant pHisStoff1 plasmid.

The Stoffel fragment DNA coding sequence was obtained by PCR amplification from *Thermus aquaticus* genomic material; ligated into the cloning vector pUC19 *Eco*RI–*Hin*dIII sites, which enabled convenient selection of the recombinant clones in a blue-white selection system. The Stoffel fragment DNA coding sequence was cut out from the recombinant pUC19Stoff plasmid with *Nde*I and *Eco*RI and in frame inserted into the expression vector pET15b *Nde*I–*Eco*RI sites (see Fig. 1 for details). Direct cloning into the expression vector would have been inconvenient due to lack of a selection system and inefficient digestion by *Nde*I close to the DNA fragment ends. Thus precloning to pUC19 plasmid was needed. The primer sequences, nucleotide and amino-acid sequences of the pHisStoff1 plasmid near the site of the Stoffel DNA fragment insertion are shown in Fig. 1. The obtained genetic construct retained the open reading frame, and the target protein contained at its N-terminus 21 additional amino acids, including a cluster of six histi-

Table 1. Purification of the His_6-tagged Stoffel fragment

Enzyme fraction	Step of purification	Total protein (mg)	Specific activity (units/mg)	Recovery (%)	Purification fold
I	Crude supernatant	190	ND	ND	ND
II	Clarified lysate after heat treatment	25	12 000	100	1
III	Eluate from metal affinity chromatography	2	30 000	98	2.5

ND, non-determined

dine residues added for purification of the recombinant protein by metal-affinity chromatography. The amino-acids sequence of the cloned Stoffel fragment is shorter by 4 amino-acid residues at N-terminus (Glu-Ser-Pro-Lys, 289–292 aa of the *Taq* DNA polymerase) than the Stoffel fragment from Perkin-Elmer.

Expression and purification of the recombinant His_6-tagged Stoffel fragment

E. coli strain BL21 (DE3) pLysS transformed with pStoff1 were grown at 37°C in 400 ml of LB containing 50 μg/ml chloramphenicol and 100 μg/ml ampicillin. The purification procedure was performed as described in part I, except that the His_6-tagged Stoffel fragment was eluted three times with 10 ml of washing buffer B containing alternatively 20, 40, 60 or 100 mM imidazole, respectively (buffers B40, B60 and B100).

Overexpression was induced by addition of IPTG to final concentration of 1mM at A_{660} of 0.3 (the best results). At $A_{660} > 0.4$ the synthesis of the recombinant protein and the activity of DNA polymerase were low.

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 resulted in precipitation of vast majority of contaminating cellular proteins allowing to obtain on a polyacrylamide gel in approximately 15% yield a single species band of about 63 kDa, corresponding to the fusion His_6-tagged Stoffel protein (Fig. 2, lane 4). For the final purification, the clarified medium was chromatographed on a Ni^{2+}-TED Sepharose column and the recombinant enzyme was eluted from the column as a single peak (about 98% purity) (Fig. 2, lanes 6–8). A band corresponding to a 63 kDa protein was observed on SDS/PAGE of crude extracts of *E. coli* BL21 (DE3) pLysS + pHisStoff 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). The pronounced enrichment in the 63 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. The amount of the 63 kDa protein, and consequently the specific activity (see Table 1), was estimated from ultraviolet absorption, 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.). After dialysis of the recombinant protein against the storage buffer, the activity of the purified His_6-tagged Stoffel fragment (25

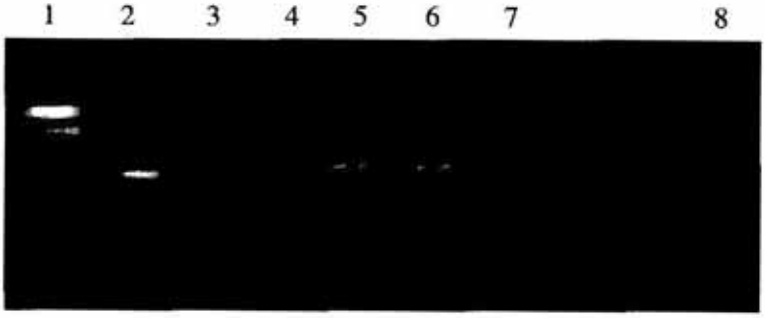

Figure 3. Monitoring of the His_6-tagged Stoffel fragment DNA polymerase activity by PCR specific reaction during the purification procedure.

Lane 1 represents the molecular mass markers (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, fraction washed out with buffer B20 (20 mM imidazole); lane 5, fraction eluted with buffer B40 (40 mM imidazole); lane 6, fraction eluted with buffer B60 (60 mM imidazole); lane 7, fraction eluted with buffer B100 (100 mM imidazole); lane 8, *Taq* DNA polymerase (1 u, Promega).

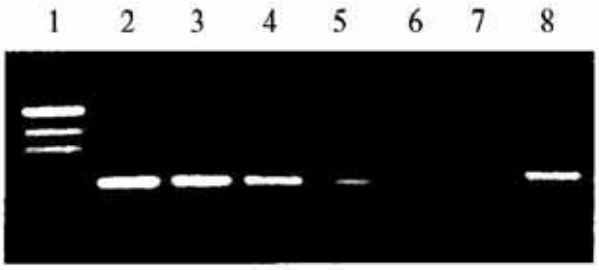

Figure 4. DNA polymerase activity of the enzyme fraction after purification by affinity chromatography on Ni^{2+}-TED Sepharose of the His_6-tagged Stoffel fragment obtained by using specific PCR amplification.

Lane 1, the molecular mass markers (501, 489, 404, 331, 242, 190, 147, 111, 110 bp); lanes 2–7, enzyme fraction of 1, 0.6, 0.4, 0.2, 0.1, 0.05 μl, respectively; lane 8, 1 u of the native *Taq* DNA polymerase (Promega).

ml) isolated from 400 ml of the *E. coli* culture was 2.4 u/μl (i.e., 150000 u/l medium) (see also Table 1).

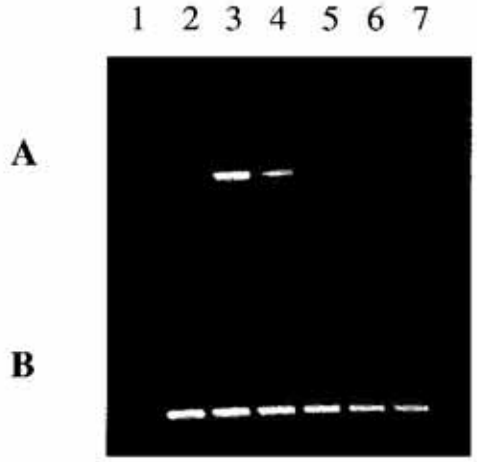

Figure 5. Temperature stability assay. Comparison of thermostability of the native *Taq* DNA polymerase (Promega) (A) and the recombinant His_6-tagged Stoffel fragment (B) using specific PCR amplification.

The enzymes were heated at 95°C before the PCR reaction for 0 min (lane 2), 1 min (lane 3), 5 min (lane 4), 10 min (lane 5), 20 min (lane 6) and 40 min (lane 7). Lane 1 represents the molecular mass markers (501, 489, 404, 331, 242, 190, 147, 111, 110 bp). PCR products were run on 2% agarose gel, stained, and photographed under UV illumination.

The relative DNA polymerase activity

The relative His_6-tagged Stoffel fragment polymerase activity was determined by compairing it with that of the native *Taq* DNA polymerase (Promega) using specific PCR amplification of the BLV *env* target (Fig. 4). The relative His_6-tagged Stoffel fragment polymerase activity was about 2.5 u/μl.

Thermostability

His_6-tagged Stoffel fragment polymerase activity was much more stable at high temperature than native *Taq* DNA polymerase. The 50% inactivation at 95°C was observed after 40 min preincubation of the purified His_6-tagged Stoffel fragment and after 10 min preincubation of the native *Taq* DNA polymerase (Fig. 5).

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