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

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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*. 

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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 Ni²⁺ 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 Ni²⁺-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 His₉-tagged proteins [15]. Cloning and expression of these His₉-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 His₉-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 Ni²⁺-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 purposes 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 His6-tagged Stoffel fragment thermostability. The studies of thermostability of the His6-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 His6-tagged Stoffel fragment.

The PCR amplification of Stoffel fragment nucleotide sequence was carried out with oligonucleotide primers (forward: HS1 5'-CGA

<table>
<thead>
<tr>
<th>293 aa</th>
<th>Ala Leu Glu Glu Ala Pro</th>
<th>5' GCCCTGGGAGGAGGCCCCC</th>
</tr>
</thead>
<tbody>
<tr>
<td>5' CGATACCATAAGCGCTGAGGAGGCCCCC 3'</td>
<td>EcoRI NdeI</td>
<td></td>
</tr>
<tr>
<td>Trp Leu Ser Ala Lys Glu Stop</td>
<td>5' TGGCTCTCCGCAAGGAGGA 3'</td>
<td></td>
</tr>
<tr>
<td>++++++++++++++++++++++++++++++++</td>
<td>Stoffel fragment N-terminus</td>
<td></td>
</tr>
<tr>
<td>3' TGGCTCTCCGCAAGGAGGA 5' reverse primer HS2</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

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

ATT CAT ATG GCC CTG GAG GAG GCC CCC, 30 nt, containing EcoRI and NdeI recog-

HindIII, was isolated from an agarose gel band using Gel-Out kit (A & A Biotechnology,
The purified fragment was ligated into pUC19 EcoRI–HindIII 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 NdeI and HindIII restriction enzymes was used as a source of Stoffel DNA fragment (1638 bp) to be cloned into the pET15b NdeI–HindIII 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 EcoRI–HindIII 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 NdeI and EcoRI and in frame inserted into the expression vector pET15b NdeI–EcoRI 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 NdeI 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 His₆-tagged Stoffel fragment

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

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 1 mM at A$_{600}$ of 0.3 (the best results). At A$_{600}$ > 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.](image)

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).
The relative DNA polymerase activity

The relative His₆-tagged Stoffel fragment polymerase activity was determined by comparing it with that of the native Taq DNA polymerase (Promega) using specific PCR amplification of the BLV env target (Fig. 4). The relative His₆-tagged Stoffel fragment polymerase activity was about 2.5 u/μl.

Thermostability

His₆-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 His₆-tagged Stoffel fragment and after 10 min preincubation of the native Taq DNA polymerase (Fig. 5).

REFERENCES


