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SHORT COMMUNICATION

Purification of recombinant Pfu DNA polymerase using a new JK110 resin

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Abstract−The purification of recombinant Pfu DNA polymerase expressed in *Escherichia coli* was studied. The lysed supernatant was heated to 75 °C to denature *E. coli* protein, followed by chromatography on JK110 and Sephadex G-75. The purified protein had comparable activity to the commercially obtained Pfu in both DNA polymerase and PCR amplification. The final product had a specific activity of 17,600 U/mg and 149,600 U of Pfu DNA polymerase was obtained from 500 ml culture. JK110 has worked well in our study and appears to be a new method of choice for purification of Pfu DNA polymerase.

Key words: Pfu, Polymerase, *Pyrococcus furiosus*, Purification, JK110

INTRODUCTION

Thermostable DNA polymerases have a crucial role in current methods of DNA amplification and sequencing [Pavlov et al., 2004]. Pfu DNA polymerase was isolated from *Pyrococcus furiosus* and shown to possess a 3'-5' exonuclease activity which enables the polymerase to proofread errors. So it is one of the best choices of PCR amplification requiring high fidelity [Cline et al., 1996]. The enzyme was initially characterized from protein isolated directly from *Pyrococcus furiosus*, but this thermophilic, anaerobic bacterium is difficult to grow to obtain large quantities of protein [Lu and Erickson, 1997]. The gene of Pfu DNA polymerase encodes a polypeptide of 775 amino acids with a predicted molecular weight of 90,109 Da [Uemori et al., 1993], and has been cloned, sequenced and expressed in various expression systems [Mroczkowski et al., 1994; Lu and Erickson, 1997; Dabrowski and Kur, 1998; Mathur, 2002]. A variety of purification systems were also developed for isolating the enzyme from expression cells. Generally, after cell growth, the purification of Pfu DNA polymerase is completed mainly by a heat-treatment and several chromatographies. Mroczkowski and Lu [Mroczkowski et al., 1994; Lu and Erickson, 1997] used a cellulose phosphate (P11) column for purification. Dabrowski [Dabrowski and Kur, 1998] purified their His-tagged Pfu by a single step metal-affinity chromatography on Ni^{2+}-Sepharose column. Mathur [2002] obtained high purity of Pfu product by a series of chromatography, including Q-Sepharose, phosphocellulose, FPLC (Mono S) and Affi-Gel Blue. In this study, we developed a new JK110 resin for purification of Pfu DNA polymerase expressed in *E. coli*. It appeared to work well and the expenditure was small.

MATERIALS AND METHODS

1. Materials

The recombinant strain was constructed by transforming the expression plasmid pETpfu (available from ATCC, Accession No. 87496) into BL21 (DE3) containing plasmids pLysS (Novagen) by using a standard protocol [Studier and Moffatt, 1986; Lu and Erickson, 1997; Novagen literature].

JK110 resin, a kind of weak acid cationic exchange resin, used in this work was provided by Shanghai Huazhen Resin Corporation (China). The resin is pretreated to Na^+ type and regenerated with 1 mol/L NaOH.

2. Purification of Pfu DNA Polymerase

Pfu was expressed in strain BL21 (DE3) pLysS carrying plasmid pETpfu. 500 milliliter (5×100 ml) LB medium plus 100 μg/ml ampicillin and 34 μg/ml chloramphenicol was inoculated with 1% overnight culture. The inoculated culture was grown at 37 °C to an OD_{600} of 0.3, then induced with 0.5 mmol/L IPTG (isopropyl β-D-thiogalactopyranoside). After being grown for another 4 h, cells were collected at 6,000 rpm for 10 min, and resuspended in 10 ml resuspension buffer (50 mmol/L phosphate, pH 7.2, 1 mmol/L EDTA). The cells were lysed with 0.5 mg/ml lysozyme at room temperature for 30 min. Then sonication was performed 5 times for 30s with a 30s interval. The mixture was centrifuged at 12,000 rpm, 30 min; then the supernatant was collected and immersed in a 75 °C water bath for 30 min. Cooled on ice for 15 min, the supernatant was collected at 10,000 rpm, 20 min.

The supernatant was then applied onto a 1.6×20 cm JK110 cation exchange column preequilibrated with 50 mmol/L phosphate, pH 7.2, 1 mmol/L EDTA for more than 10 times column volume. After loading, the column was washed intensively until the UV absorption returned to the baseline. Protein was eluted with 100 ml 0.5 mol/L NaCl prepared in 50mmol/L phosphate, pH 7.2, 1 mmol/L EDTA. Fractions were collected at 1 ml/min and assayed by using 12% SDS-PAGE [Sambrook et al., 2001]. Major fractions containing a prominent 90 kDa protein were combined and dialyzed against 50 mmol/L Tris-HCl, pH 8.2, overnight.

For further purification, the protein was concentrated and applied onto a 1.6×55 cm Sephadex G-75 (Pharmacia) gel filtration column preequilibrated with 100 mmol/L Tris-HCl, pH 8.2, 0.2 mmol/L EDTA. The fractions containing mainly 90 kDa protein were combined and stored frozen in Pfu storage buffer (50 mmol/L Tris-HCl, pH 8.2, 0.1 mmol/L EDTA, 1 mmol/L DTT, 0.1% NP-40, 0.1% Tween 20, 50% (w/v) glycerol).

3. Assay for DNA Polymerase Activity

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Pfu DNA polymerase activity was measured as described by Lu [Lu and Erickson, 1997; Mroczkowski et al., 1994]. The activity of purified polymerase was also evaluated by PCR amplification and compared to commercial Pfu DNA polymerase. The primers and a range of the purified polymerase were applied to amplify a 620 bp fragment of the template gene (plasmid, signed as BOV). The PCR reactions were done in a 20 μl mixture containing 1×Pfu buffer, 0.25 mmol/L each dNTP, 0.25 μmol/L each primer, 20 ng template, and the purified Pfu or 1 U of commercial Pfu. After amplification for 30 cycles (30s at 94 °C, 30s at 54 °C and 1.5 min at 72 °C), 5 μl of each PCR mixture was electrophoresed on a 1% agarose gel in 1×TBE.

RESULTS AND DISCUSSION

1. Purification of Pfu DNA Polymerase

Pfu was expressed as a soluble form in the cytosol. Lysozyme was added to digest the cell. To shear the DNA of *E. coli* (genomic DNA and plasmids), sonication was performed as described under the method. The vast majority of contaminating cellular proteins were denatured and eliminated by the heat treatment step (as shown in Fig. 1), using the thermostable property of Pfu. But several *E. coli* proteins still remained soluble after the heating step.

Ion-exchangers have found widespread use in the purification of proteins, due to their versatility and relative cheapness [Yao et al., 2003]. For purification of Pfu polymerase, we tried to use a new JK110 column. JK110 is a kind of even hole weak acid acrylic acid series cationic exchange resin, whose functional group is carboxyl group. The resin has an exchange capacity of more than 12.5 mmol/g (dry), water content of 70-80% and granularity scope of 0.3-1.2 mm [Chen et al., 2005; Product instruction booklet]. It is cheap and easily available in China. It also has much higher flow rate than

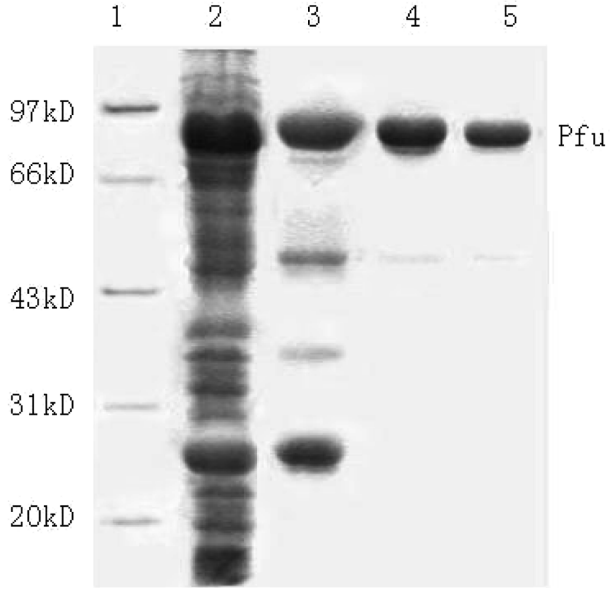

Fig. 1. SDS-PAGE analysis of the protein at each step. Lane 1, molecular weight marker; Lane 2, the protein before heat treatment; Lane 3, the protein after heat treatment; Lane 4, the eluted protein from JK110 column; Lane 5, the protein after gel filtration.

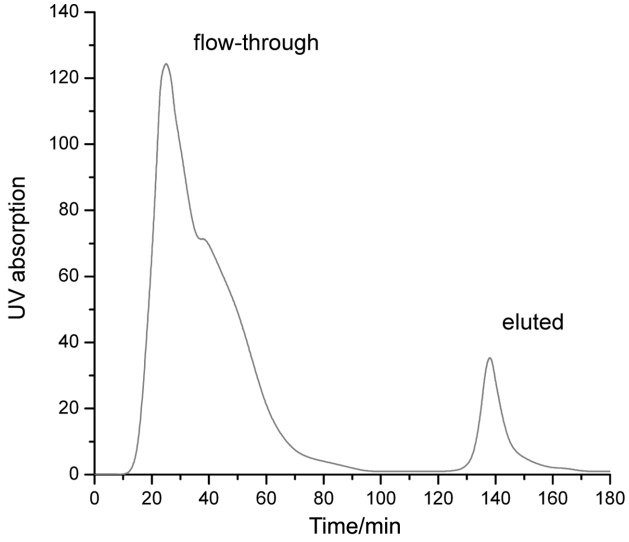

Fig. 2. The UV absorption curve of JK110 ion-exchange.

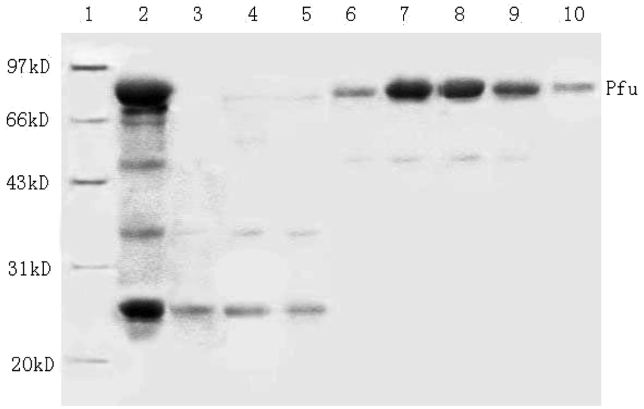

Fig. 3. SDS-PAGE analysis of Pfu purified by JK110 chromatography. Ten microliters of each fraction was applied to 12% SDS-PAGE. Lane 1, protein molecular weight marker; Lane 2, the loading sample; Lane 3-5, the flow-through fractions; Lane 6-10, the eluted fractions.

cellulose phosphate or Sepharose. The UV absorption curve of the ion-exchange is shown in Fig. 2. We can see that the flow-through fractions had a very high UV absorption, but only had comparatively slight bands in Fig. 3, suggesting that the supernatant containing large amount of non-protein substance that could cause UV absorption might be pigment or debris. Pfu was significantly purified after JK110 column (Fig. 3).

The purified product was then applied onto a Sephadex G-75 gel-filtration column for further purification. We found that the peak fractions still contained most of the weak, lower molecular weight bands seen in the loading sample, suggesting that these may be proteolytic products of Pfu itself. The gel-filtration process, in fact, gave only a small additional purification (as shown in Fig. 1). Table 1 summarizes the steps of Pfu purification.

2. DNA Polymerase Activity of the Purified Pfu

The purified Pfu was fully functional as a DNA polymerase when tested for incorporation of deoxyribonucleotides into DEAE-paper bound form. One unit of Pfu was defined as the amount of protein

Table 1. Purification of Pfu at each step

Purification step	Concentration (mg/ml)[a]	Purity (%)[b]	Pfu (mg)	Specific activity (U/mg)
Before heat treatment	29.4	13	38.1	ND[c]
After heat treatment	3.1	64	18.8	ND
JK110 chromatography	0.86	87	9.7	ND
Sephadex G-75 gel-chromatography	0.62	91	8.5	17,600

[a]The concentration of protein was determined by the Bradford assay, using BSA as the standard [Ausubel et al., 2004].
[b]Purity of Pfu at each step was estimated by analyzing digitized images of the SDS-PAGE gel using Gelpro32 software.
[c]ND, not determined.

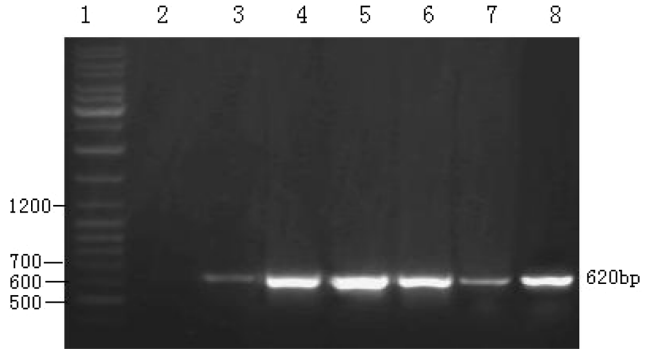

Fig. 4. Activity assay of purified Pfu in PCR. Lane 1, DNA ladder; Lane 2-7, purified Pfu of 0.2, 0.5, 1.0, 2.0, 5.0 and 10.0 U, respectively; Lane 8, 1 U of commercial Pfu from Stratagene as a positive control.

that catalyzed the incorporation of 10 nmol total nucleotide into a DEAE-bound form in 30 min at 72 °C [Lundberg and Dan, 1991; Mroczkowski et al., 1994]. The specific activity of the purified Pfu was 17,600 units per milligram of Pfu. This is comparable to the activity reported for Pfu directly purified from *P. furiosus* (31,713 units/mg) [Lundberg and Dan, 1991] or purified by Lu (22,500 units/mg) [Lu and Erickson 1997]. Using JK110, we totally obtained 149,600 U of purified Pfu polymerase from 500 ml culture£¨which was twice the amount (73,500 U) purified with cellulose phosphate (P11) by Lu [Lu and Erickson 1997].

The activity of the purified Pfu was also examined by PCR. In the PCR reaction, the purified Pfu could effectively amplify the 620 bp target gene, as shown in Fig. 4, and the same amount of purified Pfu had similar efficiency to the commercial one from Stratagene.

CONCLUSION

The recombinant Pfu DNA polymerase expressed in *E. coli* could be effectively purified by heat treatment and JK110 ion-exchange chromatography. The purified Pfu had a specific activity of 17,600 U/mg, and 149,600 U of Pfu DNA polymerase was obtained from 500 ml culture. In the PCR, the purified protein had similar amplification efficiency to the commercial enzyme. JK110 Resin has worked well in our study, and it appears to be a new method of choice for purification of Pfu DNA polymerase.

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