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Rapid and Simple Method to Prepare Functional Pfu DNA Polymerase Expressed in *Escherichia coli* Periplasm

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Abstract Pfu DNA polymerase from *Pyrococcus furiosus* was expressed in the *E. coli* periplasm, and the fully active polymerase was partially purified by applying osmotic shock, ammonium sulfate precipitation, and heat treatment. This method represents a new way of expressing and purifying functional Pfu DNA polymerase without the use of chromatography.

Key words: Pfu, periplasm, expression, osmotic

The DNA polymerase from *Pyrococcus furiosus* (Pfu) shows the lowest error rate among the thermostable DNA polymerases such as Vent, Pwo, UITma, and Taq [2, 4, 7, 8]. The low error rate is considered to result from its intrinsic 3'-5' exonuclease activity [2]. The error rate of Pfu was reported to be at least 7 times lower than that of Taq which is currently most widely used [6, 11], and 2–30 times less than those of other proof-reading polymerases [1, 2, 5]. Pfu was initially characterized with the protein purified directly from the organism, *Pyrococcus furiosus* [10]. Unfortunately, certain difficulties were encountered in growing the organism at a high temperature, therefore, the recombinant DNA technology was employed to express Pfu in *E. coli*. Lu and Erickson [9] reported a method to express and purify Pfu in *E. coli*. They used 6× His-Tag to facilitate purification. Although their purification procedure yielded a favorable result, it is rather complicated to include 2 chromatographic steps. In the present study, we describe an efficient and yet simple expression and purification of Pfu, which did not include any form of chromatography.

The primers were designed so that the PCR product could be cleaved with *Nco*I and *Sal*I. The sense primer, 5'-ctc cgc gcc atg gtt tta gat gtg gat tac ata act-3', and the antisense primer, 5'-ccg cgc gtc gac gga ttt ttt aat gtt aag cca-3', were all synthesized at Genotech (Taejon, Korea). The chromosomal DNA of *Pyrococcus furiosus* was kindly provided by Dr. Frank Jenny at the University of Georgia (Athens, GA, U.S.A.). PCR was performed with Taq DNA polymerase (Bioneer, Chungbuk, Korea) on a Mastercycler Gradient (Eppendorf, Westbury, NY, U.S.A.). The denaturation, annealing, and polymerization steps were performed at 94°C for 1 min, 50°C for 1 min, and 72°C for 4.5 min, respectively, and these steps were cycled 35 times. PCR product was purified by using a PCR Purification Kit (Bioneer, Chungbuk, Korea) and was double-digested with *Nco*I and *Sal*I. The pET22b vector (Novagen, Madison, WI, U.S.A.) was cut with *Nco*I and *Xho*I in order to insert the *pfu* gene between the *pelB* leader sequence and the 6× His-Tag. The purified linearized vector was ligated with a double-digested PCR fragment with T4 DNA ligase at 15°C for 15 h. The ligation mixture was digested with *Sal*I and brought into XL1-Blue. The transformant containing the desired plasmid was selected, and the resulting plasmid was named pET22b/pfu and brought into BL21(DE3) containing another plasmid of pLysS.

The BL21(DE3) cells containing pLysS and pET22b/pfu were both grown in a 400 ml LB medium at 37°C until OD_{600} reached approximately 1.0 when the protein production was induced by adding IPTG. The cells were harvested 3 h later by centrifugation at 4,000 rpm for 10 min. The harvested cells were resuspended in 80 ml of 30 mM TrisHCl, pH 8.0, containing 20% sucrose and 1 mM EDTA, and incubated at room temperature for 10 min with occasional swirling. The cells were collected by centrifugation at 7,000 rpm for 10 min, resuspended in an ice-cold 80 ml 5 mM $MgSO_4$, and left on ice for 10 min with occasional swirling. The cells were collected again by centrifugation at 7,000 rpm for 10 min, and the supernatant was retained. $(NH_4)_2SO_4$ was added to the final concentration of 90%, and the mixture was left at 4°C overnight. Proteins were collected by centrifugation at 15,000 rpm for 30 min, and

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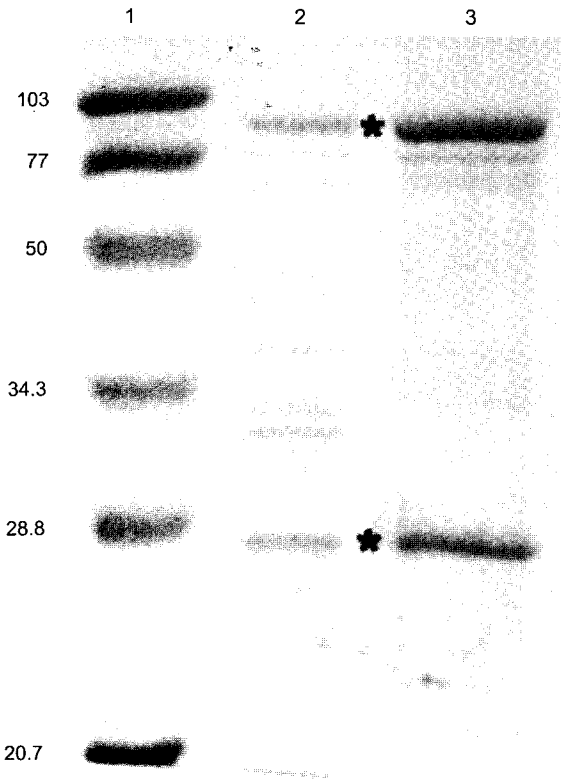

Fig. 1. *10% SDS-PAGE analysis of Pfu purification.*
Lane 1, molecular standard; lane 2, partially-purified Pfu; lane 3, fraction bound to Ni-agarose gel. See text for explanation of asterisks.

the pellet was dissolved in 3 ml of 100 mM TrisHCl, pH 8.2, containing 0.2 mM EDTA, 2 mM DTT, and 0.2% Tween 20. The resulting solution was heated at 72°C for 10 min, and the denatured proteins were spun down by centrifugation at 15,000 rpm for 15 min. The supernatant was retained, and 3 ml of glycerol was added for storage at -20°C.

The purity was checked by using a 10% SDS-PAGE gel (Fig. 1). Lane 2 in Fig. 1 shows a large band with an asterisk that corresponds to the molecular size of Pfu. It was of surprise to find that the final sample was pure enough to show the distinct Pfu band, although chromatographic procedure was not employed. To confirm that the protein band mentioned above was indeed Pfu, the cells that experienced the osmotic shock were lysed, and the soluble fraction was loaded onto the Ni-Agarose column. Since the plasmid was designed to attach a 6× His-Tag at the C-terminus of the protein, Pfu could also be purified by affinity chromatography. The bound fraction proved that the band marked with an asterisk in lane 2 was definitely identical with Pfu. Two intense bands in lane 3 indicated that the recombinant Pfu underwent some type of proteolytic cleavage inside the cells or during purification. The smaller proteolytic product did not affect the Pfu activity as described below. About 1 mg of Pfu was obtained from 400 ml LB medium, and the amount of Pfu still remained in the cytosol, i.e., about 4 mg. The efficiency to transport Pfu from the cytosol to the periplasm seemed to limit the final yield of Pfu by the preparation method described in this report. The total amount of Pfu should then be about 12 mg per liter culture, which was quite comparable to the previously reported values of 6 mg [9] and 24 mg [3].

The activity of partially purified Pfu was tested in the PCR reaction. Figure 2a shows the titration assay. The lanes 2, 3, 4, and 5 correspond to 1×, 2×, 4×, and 8× dilution of the original Pfu sample, respectively. Lane 6 shows the reaction with 5 units of commercial Taq polymerase (Bioneer, Chungbuk, Korea). The intensity of the band was analyzed with Scion Image (Scion Corporation, Frederick, MD, U.S.A.). The band intensity of lane 2 was 75% that of lane 6, which could be interpreted to indicate the Pfu concentration to be approximately 4 units/μl. Since the protein concentration was 0.16 mg/ml, the specific activity of Pfu could be calculated to be 25,000 units/mg, which is 10% higher than the published result, 22,500 units/mg [9]. The Pfu was also tested for possible production of longer PCR fragments (Fig. 2b). Lanes 2 and 3 correspond to the results with Taq and Pfu for polymerization time of 4.5 min, respectively, and lane 4 shows the result with Pfu for polymerization time of 6 min. The intensity analysis shows that the polymerization time should be at least 2 min per kb, because of the proof-reading function of Pfu.

In the present study, we showed that we developed an efficient and yet a simple procedure to produce a functional Pfu. Because this method does not rely on any form of chromatography, it is fast and convenient, and does

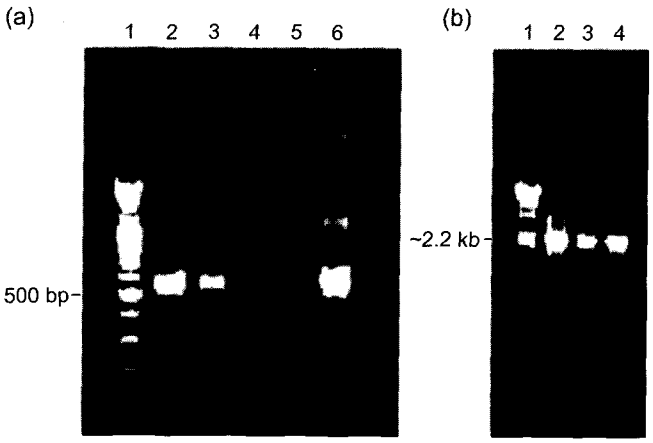

Fig. 2. Agarose gel analysis of Pfu activity assay.
(a) Lane 1, 100 bp ladder; lane 2, 1× dilution of original sample (see text); lane 3, 2× dilution; lane 4, 4× dilution; lane 5, 8× dilution; lane 6, PCR result with Taq. (b) Lane 1, molecular size marker; lane 2, PCR result with Taq (see text); Lane 3, PCR result with Pfu with polymerization time of 4.5 min; lane 4, PCR result with Pfu with polymerization time of 6 min.

not need any special equipment. The whole purification procedure takes less than 2 days, including the growth of *E. coli*, and the C-terminal 6× His-Tag may facilitate further purification, if desired. Thus, this method offers an alternative way to prepare the functional Pfu, and it has a competitive edge to the previously reported methods in terms of its speed and simplicity, combined with the ability to produce comparable amount and activity of Pfu.

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