



A SIMPLIFIED METHOD FOR THE EXTRACTION OF RECOMBINANT TAQ DNA POLYMERASE FROM *ESCHERICHIA COLI*

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ABSTRACT

DNA polymerase (Taq) enzyme isolated from *Thermus aquaticus*, a thermostable gram-negative bacterium, is a basic component of PCR, widely used in life sciences. The extraction and purification of this enzyme involves time-consuming and expensive steps such as precipitation of proteins with PEI and/or ammonium sulfate, column chromatography techniques, and removal of salts or other small molecular weight contaminants by dialysis. In this work, a novel and simplified method for extraction and purification of the recombinant Taq polymerase from *Escherichia coli* was employed, which used cold acetone instead of PEI or ammonium sulfate to precipitate the enzyme. The enzyme was efficiently recovered as active form from both the crude cell lysate and column fractions with cold acetone precipitation. This simplified method enabled us to obtain high quality Taq DNA polymerase in a much shorter time and at a lower cost.

Keywords: Taq DNA polymerase, Acetone precipitation, PCR, *Thermus aquaticus*

INTRODUCTION

A thermostable DNA polymerase enzyme isolated from *Thermus aquaticus* (Taq DNA polymerase) is an essential enzyme of the polymerase chain reaction (PCR) routinely used in all disciplines related to biological sciences. The Taq DNA polymerase was first isolated and characterized from *T. aquaticus*, a thermophilic gram-negative bacterium, in 1976 by Chien *et al.* (1976). This DNA polymerase was applied in *in vitro* DNA synthesis works in the mid-1980s (Mullis *et al.*, 1986; Mullis and Faloona, 1987; Saiki *et al.*, 1985) and enabled the development of the PCR techniques. Since *T. aquaticus*, the source of Taq DNA polymerase, is living in extreme environments and requires special growth conditions (Ray *et al.*, 1971; Ohtani *et al.*, 2010), this has led to the recombinant production of this enzyme in *E. coli* cells, making it much easier and cheaper.

The gene encoding the DNA polymerase enzyme of *T. aquaticus* has first been cloned into *E. coli*, and it has been shown that this gene is effectively involved in expression in these cells by Lawyer *et al.* (1989). The production of recombinant Taq DNA polymerase in *E. coli* has greatly reduced the cost of the enzyme that is great importance especially for laboratories that routinely work based on PCR techniques. While there are some minor differences, a similar procedure is generally followed for the extraction and purification of the recombinant Taq DNA polymerase from *E. coli* cells. Considering the works related to the subject, it is seen that the methods used for purification of Taq DNA polymerase mainly consist of lysis of the cells, precipitation of DNA-bound or non-bound proteins with polyethylenimine (PEI) and/or ammonium sulfate, removal of salts or other undesirable small molecules by dialysis, and protein purification with various column chromatography techniques.

Engelke *et al.* (1990) had suspended the bacterial cells producing enzyme in lysis buffer, lysed at 75 °C for 1 hour, and then precipitated Taq DNA polymerase in the lysate by adding PEI at final concentrations of 0.50-0.80% and subsequent centrifugation. After suspending the precipitate in a different buffer, they had repeated the PEI precipitation process several times. After dialyzing the extract, they had passed it through BioRex 70 ion exchanger (Bio-Rad) column to collect fractions containing Taq DNA polymerase enzyme. The methodology of Engelke *et al.* has also provided the base for many other studies (Grimm and Arbuthnot, 1995; Lawyer *et al.*, 1993). Pluthero (1993) had precipitated proteins including Taq DNA polymerase from the cell lysate with ammonium sulfate, followed by dialysis processes and chromatographic separation techniques. Ishino *et al.* (1994) had disrupted *E. coli* cells by sonication, precipitated Taq DNA polymerase enzymes in lysate

with both ammonium sulfate and PEI, and subsequently exploited chromatographic techniques. Recently, Chen *et al.* (2015) have succeeded in recovering of Taq DNA polymerase from the cell lysate by ethanol precipitation.

The precipitation of Taq DNA polymerase enzyme with PEI and/or ammonium sulfate, removal of salts with dialysis processes before and after column chromatography, and column chromatography techniques are time-consuming and costly applications. On the other hand, concentrating the Taq DNA polymerase in the fractions collected from the column requires additional processes. In this work, the recombinant Taq DNA polymerase synthesized in *E. coli* was efficiently isolated in a simpler way, a very short time and at low cost with cold acetone precipitation method.

MATERIAL AND METHODS

Escherichia coli and plasmid

E. coli BL21 (DE3) cells were used as host bacterium. The pAKTaq plasmid DNA carrying the DNA polymerase gene of *T. aquaticus* (Engelke, *et al.*, 1990) was obtained from Addgene.

Preparation of crude bacterial lysate

Competent *E. coli* BL21 (DE3) cells were prepared according to the method described by Inoue *et al.* (1990) and transformed with pAKTaq plasmid DNA. A single colony was inoculated into 5 ml of LB medium (+amp) and, it was incubated overnight with shaking (180 rpm) at 37 °C. The pre-culture was inoculated into 150 ml of fresh LB medium (+amp) and the cells were grown for 8-10 hours at 37 °C, in shaking incubator (180 rpm). The cells were collected by centrifugation at 8000 rpm, for 10 minutes at room temperature. The cell precipitate was suspended in 10 ml of buffer A (50 mM Tris-HCl, 50 mM Dextrose, 1 mM EDTA, 2 mM PMSF, pH 8), added lysozyme at 1 mg/ml final concentration and, incubated at room temperature for 15 minutes. Subsequently, 10 ml of lysis buffer (10 mM Tris-HCl, 50 mM KCl, 0.5% Tween-20, 1 mM EDTA, pH.8) was added to the cell suspension and incubated for 60 minutes at 75 °C to lysis of the cells. Non-soluble cellular residues were removed by centrifugation at 15,000 rpm for 20 minutes, at room temperature. The supernatant was used for Taq DNA polymerase extraction with cold acetone or PEI.

Preparation of samples by precipitation with acetone and PEI

In small volumes of enzyme extraction, 250 µL crude supernatant was mixed with cold acetone at ratios of 1/1, 1/2, 1/3, 1/4 or 1/5 and, stored at -20 °C for 15-20 minutes. The enzyme in the samples was precipitated by centrifugation at 15,000 rpm for 20 min, at 4 °C. The precipitates were dried at room temperature for 5 minutes and suspended in 10 µL of storage buffer (50 mM Tris-HCl, 50 mM KCl, 0.1 mM EDTA, 1 mM DTT, 2 mM PMSF, 50% Glycerol, pH: 7.9) or buffer A. Insoluble substances were removed by centrifugation at 15,000 rpm for 5 minutes, at 4 °C and, then supernatants were used for Taq enzyme activity assays or SDS-PAGE analyzes. To precipitate Taq DNA polymerase with PEI, 10% PEI solution was added to 250 µL crude supernatant at final concentrations of 0.1%, 0.25%, 0.5%, or 1%, and the samples were centrifuged at 15,000 rpm for 20 minutes, at 4 °C. The precipitates were suspended in 10 µL of storage buffer or buffer A and then used in subsequent tests.

Gel filtration column chromatography

Sephadex G-50 gel filtration column (15x200 mm / 35 mL) was used for purification of Taq DNA polymerase. The gel matrix was swollen in buffer A containing 0.01% NaN₃. The column was equilibrated with same buffer. The proteins in 5 ml of the crude lysate were precipitated with 3 volumes of cold acetone. The precipitate was dried at room temperature for 5 minutes and suspended in 0.5 ml buffer A. The insoluble part was removed by centrifugation at 15,000 rpm for 5 minutes, at 4 °C, and the supernatant was applied to the column. The column fractions were collected with a fraction collector (25 drops/tube). The optical densities of the fractions were determined by spectrophotometer at 253 nm wavelength. To detect the enzyme activity in column fractions, Taq polymerase in 250 µL of each fraction was precipitated with 3 volumes of cold acetone. The precipitates were dried at room temperature for 5 minutes, dissolved in 10 µL storage buffer and used for PCR.

Activity tests and protein analysis

Taq DNA polymerase activities of the samples prepared as mentioned above, were determined by standard PCR that is performed in 20 µL reaction volume. The enzyme activities in samples were compared with a known commercial Taq DNA polymerase. The proteins and Taq DNA polymerase enzymes in the samples were analyzed by denaturing SDS-PAGE (5% stacking gel, 10% running gel). After electrophoresis, the proteins and/or Taq DNA polymerase were visualized with silver staining or Western blotting by using monoclonal anti-TaQ antibody (Mybiosource MBS430075).

RESULTS AND DISCUSSION

In this work, the Taq DNA polymerase in the crude cell lysate was precipitated by using both cold acetone and PEI at different ratios, and the enzyme activity in the precipitates was determined by PCR. The results of PCR performed with the enzyme samples are given in Figure 1A. DNA synthesis activity was observed with enzyme sample prepared with acetone precipitation (a-TaQ), but not in samples prepared with PEI precipitation. The activity of a-TaQ was compared with a known commercial enzyme (c-TaQ). As shown in Figure 1C, the a-TaQ DNA polymerase was found to be as active as the c-TaQ DNA polymerase.

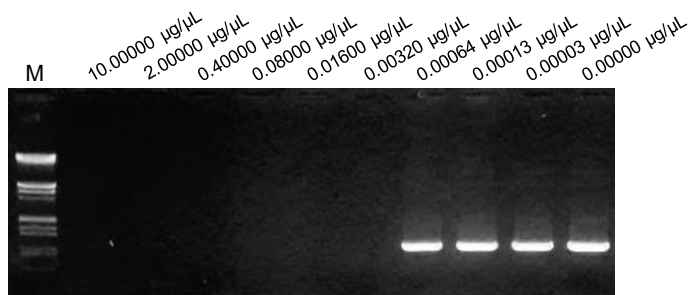


Figure 2. The effect of PEI on the enzymatic activity of Taq DNA polymerase. The PCR was performed with 1 µL (~ 5U) of Taq DNA polymerase enzyme (a-TaQ) prepared with cold acetone as indicated in Figure 1A. M. Lambda DNA Marker (EcoRI/HindIII).

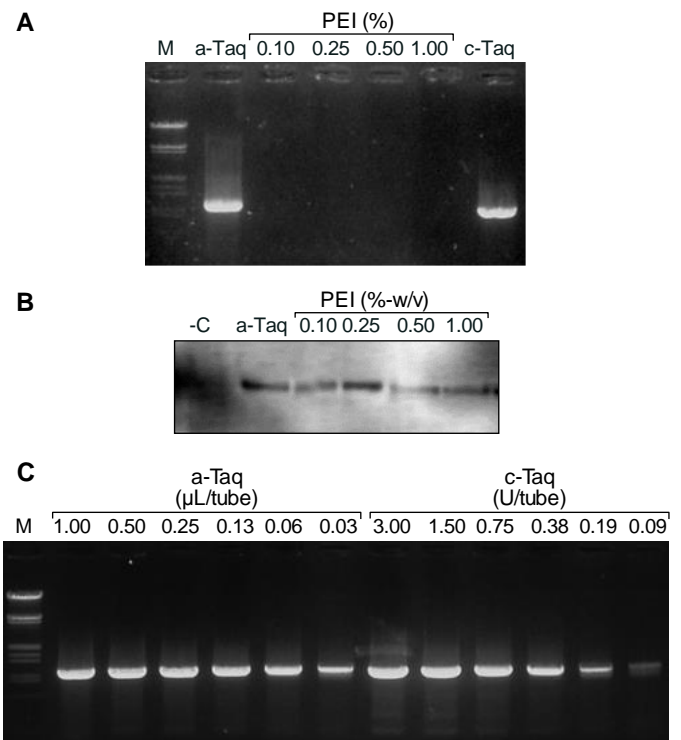


Figure 1. A. The activity of Taq DNA polymerase enzyme prepared by precipitation with cold acetone or PEI. Taq DNA polymerase samples were prepared from 250 µL of the crude cell lysate as described in the methods with mixing 3 volumes of cold acetone (a-TaQ) or PEI at the concentrations indicated on the figure. The precipitates were then dissolved in 10 µL of storage buffer and used for the PCR. B. Western blot analysis of Taq DNA polymerase enzyme in the samples. C. Comparison of the a-TaQ with commercial Taq polymerase (c-TaQ). M. Lambda DNA Marker (EcoRI/HindIII).

Since enzymatic activity was not detected in samples prepared with PEI, it was investigated whether the samples contain the Taq DNA polymerase with Western blotting. As shown in Figure 1B, the Taq polymerase was detected in all samples. Therefore, it was thought that the reason of inability to detect enzymatic activity in the samples might be due to PEI contamination. To reveal this, the effect of PEI on Taq DNA polymerase (a-TaQ) activity was tested by adding different amounts of PEI in reaction medium. As shown in Figure 2, PEI has completely blocked the enzyme activity at 2-3 µg/ml concentration (0.0032 + 0.00064/2 = 0.00192 µg / µL) in the medium. Even if PEI is not a direct inhibitor of Taq DNA polymerase, it has been shown that the presence of small amounts of PEI in the medium blocks the enzymatic reaction. Therefore, PEI should be completely removed from the medium by dialysis and/or column chromatography techniques.

The ratio of acetone required for maximum recovery of Taq DNA polymerase from the crude lysate was investigated by using 1-5 volumes of acetone. Even an equal volume of acetone was found to be sufficient to recover high ratios of Taq DNA polymerase from crude lysate (Figure 3).

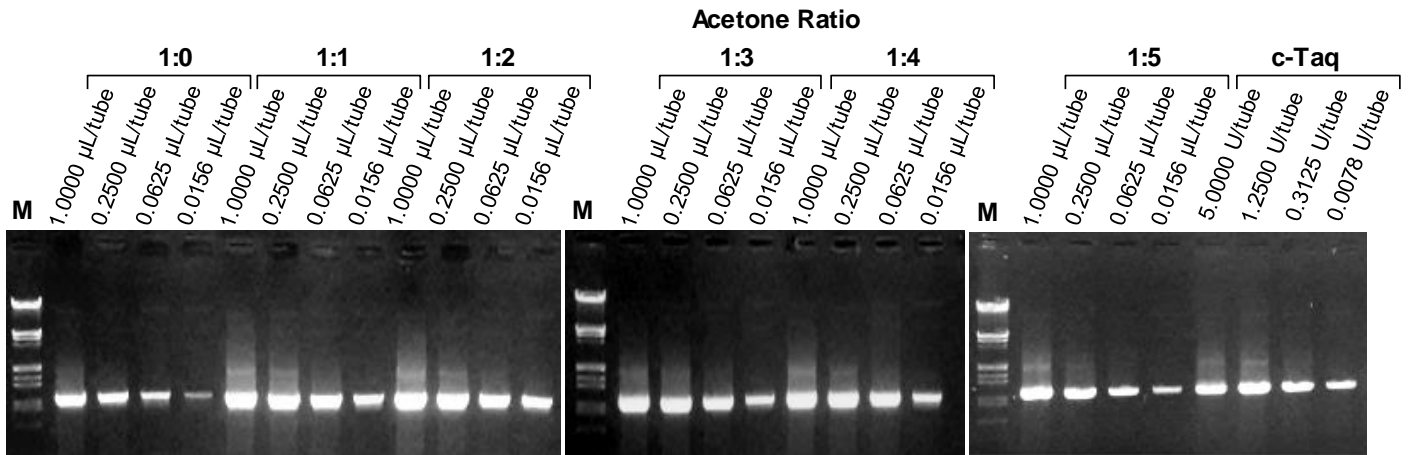


Figure 3 Taq DNA polymerase activities in samples precipitates with different volumes of acetone. Samples were prepared by mixing of 250 μ L crude supernatant with acetone at the ratio indicated on the figure. The resulting precipitates were dissolved in 10 μ L of storage buffer and used in the PCR as indicated amounts. c-Taq. Commercial Taq. M. Lambda DNA Marker (EcoRI/HindIII).

On the other hand, the amounts of Taq DNA polymerase in samples precipitated with acetone were analyzed by denaturing SDS-PAGE followed by Western blotting or silver staining. The results showed that at least 2 volumes of acetone is necessary to achieve the maximum efficiency (Figure 4).

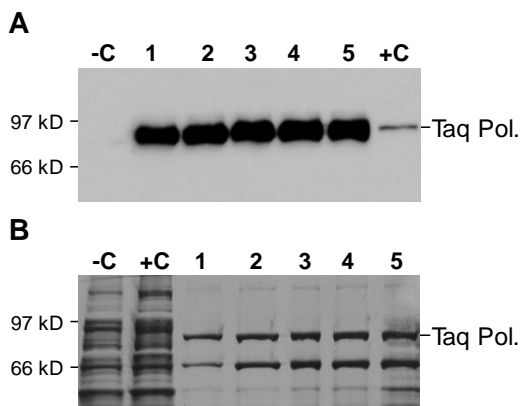


Figure 4. SDS-PAGE analysis of the Taq DNA polymerase in the samples precipitated with cold acetone at different ratios (1. 1/1; 2. 1/2; 3. 1/3; 4. 1/4; 5. 1/5). **A.** Western blotting. **B.** Silver staining. The samples were prepared by mixing 250 μ L of crude lysate with acetone at the indicated ratios. The precipitates were suspended in 20 μ L buffer A, mixed with equal volumes of x2 SDS-sample buffer and applied 10 μ L of samples to each well.

The concentrating of Taq DNA polymerase by precipitation with cold acetone also provides an easily applicable way for recovering of the enzyme from column fractions without dialysis processes. A sample prepared by precipitation with acetone as described in the methods and dissolved in 0.5 ml buffer A was applied to the Sephadex G-50 Gel filtration column (Figure 5A). The proteins in column fractions were precipitated by mixing with 3 volumes of cold acetone and, enzyme activities in the precipitates were determined by standard PCR. It was observed that the Taq DNA polymerase enzyme was eluted from the column in fractions 6-9. (Figure 5B). The enzyme activity in fraction seven was found as high as that of commercial Taq DNA polymerase (Figure 5C).

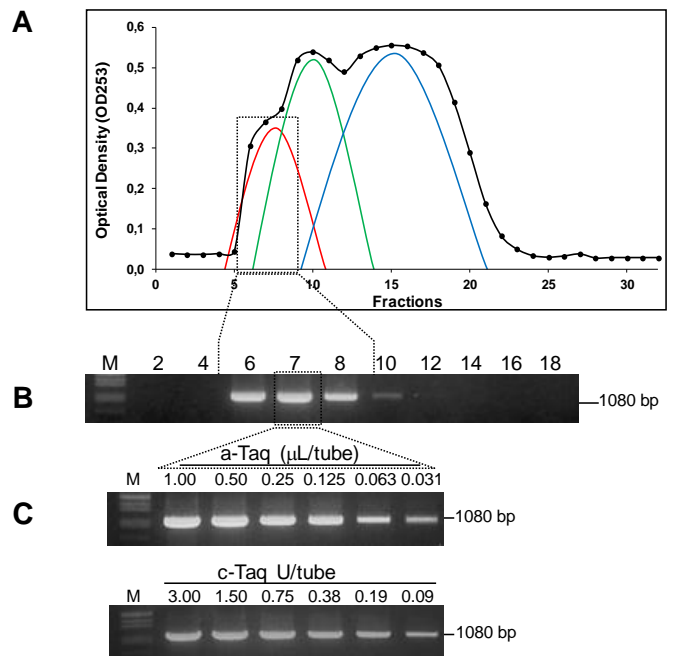


Figure 5 Sephadex G-50 gel filtration column chromatography of sample prepared with acetone precipitation (A). The activities of Taq DNA polymerase in the fractions (B). Comparison of Taq polymerase (a-Taq) activity of fraction 7 with commercial Taq polymerase (c-Taq) (C). Samples were prepared by mixing 250 μ L of fraction with 3 volumes of cold acetone. The precipitates were suspended in 10 μ L of storage buffer and used in the PCR. Column chromatography was carried out at room temperature (20-22 $^{\circ}$ C). Column dimensions: 15x200 mm; Fractions: 25 drops / tube (~ 2 ml); Mobile phase: buffer A containing 0.02% NaN_3 . M. Lambda DNA Marker (EcoRI/HindIII).

While general extraction procedures for isolation and purification of enzymes/proteins are applicable to all organisms, it is invariably necessary to develop unique strategies for isolation of the target protein of interest. Proteins have amphoteric properties. Therefore, several methods are available for precipitation of proteins utilizing changes in pH and temperature, or addition of salts, polymers and organic solvents (Burgess, 2009; Scopes, 2007). The precipitation with PEI and/or ammonium sulfate is frequently employed for separation of the Taq DNA polymerase from cell lysates. Polyethylenimine is a positively charged polymer, and forms complexes with nucleic acids (Utsuno and Uludag, 2010). Therefore, it has been used for isolation of DNA/RNA-binding proteins or enzymes (Burgess, 1991; Marenchino, Armbruster and Hennig, 2009). PEI is also used for gene transfer to mammalian cells as a non-viral gene carrier, because of its ability to form complexes with DNA molecules

(Baker *et al.*, 1997; Çağlayan and Turan, 2016). PEI has been used for Taq DNA polymerase extraction in previous studies (Engelke, *et al.*, 1990), and usually removed by dialysis and ion exchange column chromatography techniques. In this study it was shown that the presence of very small amounts of PEI in samples strongly blocks Taq enzyme activity (Figure 2). Therefore, PEI should be thoroughly eliminated from the samples. These processes take a long time and increase the cost. In contrast, in the case of the Taq enzymes prepared with acetone precipitation, but not fully purified, enzyme activity has not been inhibited (Figure 1A).

Ammonium sulfate is the most commonly used precipitant for salting out of proteins. It precipitates most proteins at maximum saturation (3.8 M at 0°C and 4.1 M at 25°C), and protects proteins in solution from denaturation (Burgess, 2009; England and Seifter, 1990). The use of ammonium sulphate at different saturation allows for a certain degree of separation of the proteins. After the salting out process, the residues of salts in the samples require to be removed with dialysis. Different organic solvents are also being tested for the precipitation of proteins. Some of these can cause protein denaturation and/or destabilization (Lehninger, 1993). For this reason, the salting out technique is preferred especially when the enzymes are precipitated.

In this work, which we have undertaken with acetone, an organic solvent, the thermostable Taq DNA polymerase enzyme activity is maintained. The samples prepared with acetone precipitation can be dried shortly at room temperature, dissolved in appropriate buffer and used for PCR without additional processes. In the same way, the Taq DNA polymerase in the fractions collected by column chromatography can be concentrated with cold acetone. Chen *et al.* (2015) have used ethanol to extract of Taq polymerase form lysates in a similar study. Precipitation with acetone has more advantageous than ethanol in terms of time and cost. One of the most important problems encountered in Taq enzyme extraction is nucleic acid contamination. Ethanol is an ideal organic solvent for precipitating the nucleic acids and is used for routine DNA isolation studies. In this respect, precipitation of Taq polymerase from cell lysates with acetone offers an important advantage.

CONCLUSION

The Taq DNA polymerase having a commercial value is widely used in PCR assays. In this study, we presented a simple and inexpensive method for extraction and purification of recombinant Taq DNA enzyme from *E. coli* cells. The Taq DNA polymerase was easily recovered from both the crude bacterial lysate and the column fractions by precipitation with cold acetone. It is thought that this method will allow the production of the Taq DNA polymerase enzyme at lower cost.

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